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Cues for Cancer Stem Cells Origin

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Cues for cancer stem cells origin

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ABSTRACT

Neural stem/progenitor cells (NSPC) can differentiate into neurons and glial cells in the central nervous system. Interestingly, NSPC biology is being applied to the study of human brain tumours, since these cells share some common features with glioma cells. However, it is not known the developmental stage with more similarities to glioma cells, or the most susceptible to malignant transformation.

We aimed to identify the stage(s) in the NSPC differentiation process towards astrocytes where cells acquire phenotype characteristics comparable to glioma cells.

NSPC that were obtained from E15 mouse brain, were grew as neurospheres (NS) and induced to astroglial differentiation until 7 days *in vitro* (DIV). After the cellular characterization of NS and differentiating cells, tumour-related factors were evaluated and their behavior compared to the one of GL261 mouse glioma cells.

Astroglial differentiation led to a decrease in progenitor cells, as expected. Multidrug resistance-associated protein 1 expression decreased and autophagy marker increased with differentiation. The vascular endothelial growth factor (VEGF), matrix metalloproteinases and S100B protein increased until 2/3 DIV, while the 1 DIV cells showed the highest migratory potential towards the chemotactic VEGF or GL261-conditioned media.

Comparison of data with glioma cells characteristics point to the first and second days of NSPC differentiation to astrocytes as the stages closing matching GL261 cells, and likely the most vulnerable to malignancy transformation.

Keywords: Astrocytes, Neural progenitor cells, Neural stem cells, Glioma cells, Gliomagenesis, Tumour-related factors

RESUMO

As células estaminais/progenitoras neurais (CEPN) podem diferenciar em neurónios e células da glia no sistema nervoso central. A biologia das CEPN tem sido aplicada ao estudo dos tumoures cerebrais humanos, uma vez que estas células partilham algumas características com as células de glioma. Contudo, não é conhecido o estadio do desenvolvimento mais semelhante às células de glioma, ou o mais susceptível à transformação maligna.

O nosso objectivo é identificar o(s) estadio(s), no processo de diferenciação das CEPN em astróctios, no(s) qual(is) as células adquirem características fenotípicas comparadas às células de glioma.

As CEPN, obtidas de cérebros de embriões de ratinho no 15º dia de gestação, foram cultivadas como neuroesferas e induzidas à diferenciação astroglial até 7 dias *in vitro* (DIV). Após a caracterização celular das neuroesferas e células em diferenciação, foram avaliados determinados factores tumorais e o seu comportamento comparativamente às células de linha celular de glioma de ratinho (GL261).

A diferenciação astroglial levou ao decréscimo das células progenitoras, como esperado. A expressão da proteína associada à resistência a multidrogas decresceu, enquanto a autofagia aumentou ao longo da diferenciação. O factor de crescimento endotelial vascular (VEGF), metaloproteinases e a proteína S100B revelaram um aumento da expressão até 2/3 DIV. Ainda, o fenótipo correspondente a 1 DIV em condições de diferenciação foi o que apresentou maior potencial migratório para o VEGF ou para o meio proveniente das células de glioma.

A comparação dos dados obtidos para os vários factores, levou-nos a sugerir o primeiro e segundo dias das CEPN em condições de diferenciação em astrócitos, como os estadios mais próximos das células de glioma, e por consequente, mais vulneráveis a transformação maligna.

Palavras-chave: Astrócitos, Células estaminais neurais, Células progenitoras neurais, Células de glioma, Fatores tumourigénicos, Gliomagénese.

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ABBREVIATIONS

AB/AM	Antibiotic antimycotic solution
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
BBB	Blood brain barrier
bFGF	Basic fibroblast growth factor
Bmi-1	B-cell-specific Moloney murine leukemia virus integration site 1
BMP	Bone morphogenetic proteins
BrdU	5-Bromo-2'-Deoxyridine
BSA	Bovine serum albumin
CEPN	Células estaminais/progenitoras neurais
CNS	Central nervous system
CSC	Cancer stem cells
DAPI	4',6-diamidino-2-phenylindole
DIV	Days *in vitro*
Dlx2	DLX2 gene (protein coding) distal-less homeobox 2
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNAse I	Deoxyribonuclease I
E	Embryonic day
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
HBSS	Hanks' balanced salt solution without Ca^{2+} and Mg^{2+}
LC3	Microtubule-associated protein light chain 3
MAP2	Microtubule-associated protein 2
MBP	Meylin basic protein
MMP	Matrix metalloproteinases
Mrp1	Multidrug resistence-associated protein 1
NEP	Neuroepithelial progenitor cells
NG2	Neural/glial antigen 2
NP	Nanoparticles

NPC	Neural precursor cells
NS	Neurospheres
NSC	Neural stem cells
NSPC	Neural stem/progenitor cells
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PDGFRα	Platelet-derived growth factor receptor α
PDL	Poly-D-lysine
PFA	Paraformaldehyde
PI	Propidium iodide
PMSF	Phenylmethylsulfonyl fluoride
PTEN	Phosphatase and tensin homolog
RGC	Radial glial cells
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate-polyacrilamide gel electrophoresis
SGZ	Subgranular zone
Sox2	SRY (sex determining region Y)-box 2
SVZ	Subventricular zone
TMZ	Temozolomide
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor 2
VZ	Ventricular zone
WHO	World Health Organization

I. INTRODUCTION

1. Neural Stem Cells

The term “stem cell” was originally proposed by Heackel in 1868 as cited by Breunig *et al.* (2011). Stem cells are a class of undifferentiated cells with the ability to differentiate into specialized cell types. They can also generate new stem cells by self-renewal and can be classified according to their sources: embryonic stem cells (that arise from the blastocyst phase of embryonic development) and adult stem cells (that arise from adult tissue). Stem cells can give rise to several cell types with more limited self-renewal and proliferation ability, including neural stem cells (NSC).

NSC only became popular in the early 1990s (Breunig *et al*., 2011) and are described as a brain population with the ability of self-renewal, capable to maintain a pool of neural stem-like cells (extensive proliferative potential). These cells can also differentiate into more restricted precursor cells, designated by neural precursor cells (NPC) that are able to produce the three major cell types that compose the central nervous system (CNS): neurons, astrocytes and oligodendrocytes (Gage, 2000; Siebzehnrubl *et al*., 2011; Temple, 2001) (Fig. I.1).

NSC present a promising therapeutic tool for brain disorders, as they might be used to replace virtually any type of neuron lost from neurodegenerative disords such as Parkinson’s disease (PD), Alzheimer’s disease (AD) and amyotrophic lateral sclerosis (ALS) (Breunig *et al*., 2011; Parish *et al*., 2008). Interestingly, NSC show tropism for brain tumours, namely gliomas and thus, they can be used as carriers for anti-tumourigenic drugs (Aboody *et al*., 2000; Noble, 2000). However, if in one hand NSC may have a therapeutic potential, on the other hand it is suggested that these cells might generate brain tumours, due to their high proliferative potential. Hence, it is very important to understand the mechanisms by which NSC generate the diversity of their resulting progeny (Breunig *et al*., 2011; Vescovi *et al*., 2006).

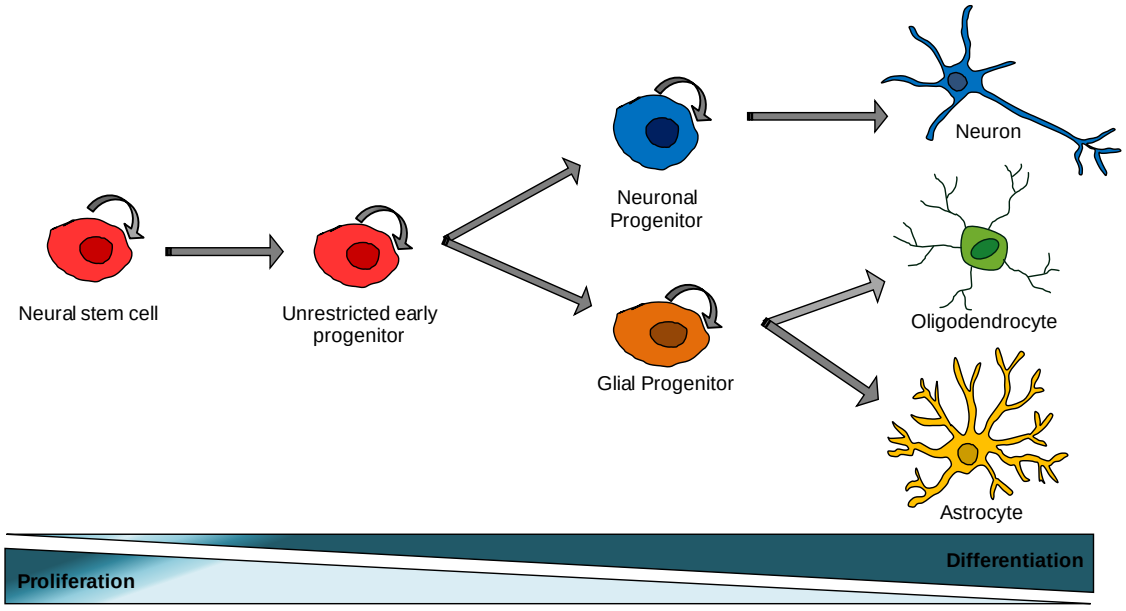


Fig. I.1 – Classical view of neural stem cells hierarchy. The normal neural stem cell production of progenitor cells, which subsequently generates the three differentiated cell types of the central nervous system: neurons, oligodendrocytes and astrocytes (trilineage potential). Along the differentiation process, the proliferative potential decrease, while the number of differentiated cells increase.

1.1. NSC in central nervous system development

The CNS of mammals is a highly complex structure made up of a huge number of neurons, glial cells and synapses, all linked by extremely heterogeneous anatomical and functional relationships. This complex and heterogeneous cellular population derives from NSC or primary progenitors (Bonfanti and Peretto, 2007; Merkle and Alvarez-Buylla, 2006). Very early in the mammalian development, the CNS begins with the development of the neuroectoderm, which forms the neural plate [at embryonic day 7.5 (E7.5) in mice] and then folds, giving rise to the neural tube (at E8.5 in mice) (Conti and Cattaneo, 2010; Merkle and Alvarez-Buylla, 2006; Temple, 2001). Within this primitive neural structure, a complex and heterogeneous population of NSC and primary progenitors can be found, the neuroepithelial progenitor cells (NEP). NEP are radially elongated and contact with both the apical (ventricular) and basal (pial) surfaces of the embryonic brain (Merkle and Alvarez-Buylla, 2006) expressing neural precursor markers such as Sox1 [SRY (sex determining region Y)-box 1] and Nestin, due to their "stemness" properties (Conti and Cattaneo, 2010). During the development course, these cells undergo both symmetric and asymmetric types of division. On the first stage of neural development, NEP undergo symmetrical divisions to expand the neural stem cell pool (self-renewal and proliferative potential), while in the second stage, they initiate asymmetrical division to generate a stem cell (that remains in the ventricular zone - VZ) and a daughter cell, that migrates radially outward to its final position in the brain (intermediate progenitor) (Farkas and Huttner, 2008; Merkle and Alvarez-Buylla, 2006).

In a later stage, NEP also originate the radial glial cells (RGC) (around E9.5 in mice) (Bentivoglio and Mazzarello, 1999; Bonfanti and Peretto, 2007; Conti and Cattaneo, 2010) which are the principal primary progenitors of the mammalian embryonic forebrain (and early postnatal brain). Similarly to NEP, RGC divide in the VZ and maintain contact with the pial surface via a radially projecting basal process. Thus, it is thought that NEP transform directly into RGC, which are the main cell type in the developing brain. They are considered an important transient population once RGC function both as neural progenitors and a scaffold for migrating immature neurons (Conti and Cattaneo, 2010). RGC express glial/astroglial markers as the glutamate aspartate transporter (GLAST), the glial fibrillary acidic protein (GFAP) and the brain lipid binding protein (BLBP) (Farkas and Huttner, 2008; Merkle and Alvarez-Buylla, 2006) and have astroglial cells anatomical-like features, such as endfeet on blood vessels, intermediate filaments, and glycogen granules (Merkle and Alvarez-Buylla, 2006). RGC can undergo symmetrical proliferative or asymmetrical neurogenic divisions to generate neurons (the functional unit of the nervous system), as well astrocytes and oligodendrocytes. These cells provide a critical support role for optimal neuronal functioning and survival but its differentiation potential is less broad than that of NEP (Merkle and Alvarez-Buylla, 2006; Zhao *et al*., 2008). Hence, NEP and RGC comprise the first group of stem and progenitor cells (apical progenitors - AP) of the CNS. It is still important to refer that, in mammalian, RGC disappear from the brain soon after birth, giving place to a second group composed by those neural progenitors that undergo mitosis in the basal VZ and subventricular zone (SVZ) (basal progenitors – BP) (Bonfanti and Peretto, 2007; Farkas and Huttner, 2008; Merkle and Alvarez-Buylla, 2006) (Fig. I.2).

The SVZ, a region just above the ventricular zone (VZ), remains the stem character in the adult brain. Particularly, once RGC and SVZ astrocytes share many properties, it is though that both belong to the same lineage. Moreover, RGC of the neonatal lateral ventricular wall occupy the same region as the astrocytic stem cells of the adult SVZ, and they act as multipotent NSC either *in vitro* or *in vivo*. SVZ cells also retain a radial process and express GFAP, suggesting that RG cells may directly transform into SVZ astrocytes, in adult brain. In summary, current evidences suggest that NSC gradually transform from NEP to RGC and from these to astrocytes-like cells (Merkle and Alvarez-Buylla, 2006).

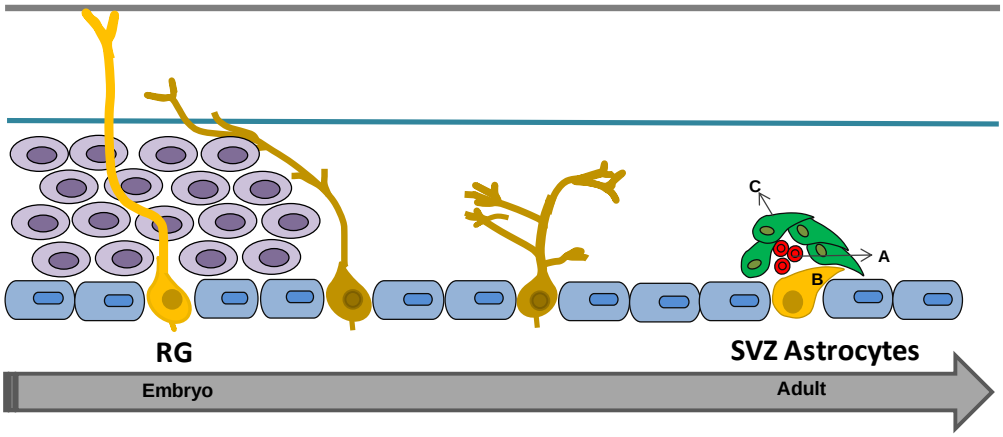


Fig. I.2 – Gradual transformation of radial glial cells into astrocytes-like cells, from the embryonic period to adulthood. Fate of radial glial (RG) cells from development (left) to adulthood (right). Different degrees of yellow indicate progressive maturation from RG to subventricular zone (SVZ) astrocyte-like cells, also called neurogenic SVZ astrocytes. In the embryo, RG cells behave as multipotent stem cells. In brain parenchyma they transform into SVZ astrocytes through a transient unipolar form which has lost contact with both pia mater and ventricular surface (in grey: ependyma). The SVZ is composed by type B cells – SVZ astrocytes stem cells (yellow), type C cells – transient-amplifying cells (green) and type A cells - neuroblasts (red) , such as described in the next section. Hence, it is thought that RG cells (in the embryo) disappear and originate SVZ astrocytes (in the adult). Adapted from Bonfanti and Peretto (2007).

1.2. NSC in the adult brain niches

Neurogenesis in the adult mammalian CNS was first described in the 1960s (Siebzehnrubl *et al*., 2011; Till and Mc, 1961). Adult neurogenesis is maintained by NSC that persist in the adult mammalian brain and undergo self-renewal and have multipotency capacity generating neurons and macroglia (astrocytes and oligodendrocytes). Interestingly, it is thought that this adult neurogenesis might be related with malignant processes giving rise to adult brain tumours (Riquelme *et al*., 2008).

Two germinal regions are found in the adult mammalian brain: the SVZ of the forebrain lateral ventricle (Fig. I.3 A) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Fig. I.3 D) (Doetsch, 2003; Riquelme *et al*., 2008; Siebzehnrubl *et al*., 2011). These two zones are known as neurogenic niches for adult stem cells because their microenvironment regulate and support self-renewal, activation and differentiation (features of stem-like cells) (Doetsch, 2003; Riquelme *et al*., 2008). Also, they have several architectural elements that contribute to the adult neurogenesis such as extensive cell-cell interactions, proximity to the cerebrospinal fluid of the lateral ventricle, close

association with blood vessels, rich extracellular matrices and specialized basal lamina (Doetsch, 2003). Therefore, they are exposed to a variety of growth factors (Tavazoie *et al*., 2008). Recent data suggest that a major property of several stem cell niches is the intimate association with endothelial cells, which regulate stem cell self-renewal and differentiation (Riquelme *et al*., 2008).

In the SVZ, three types of neural progenitors can be identified, type A, B and C cells (Fig. I.3 B). Type B cells are radial glia-like cells located in the subependymal layer. Type B cells are also designated as SVZ astrocytes stem cell progenitors, because they express vimentin, nestin and GFAP, which divide and generate transient amplifying progenitors [type C cells; $GFAP^-$/ distal-less homeobox 2 $(Dlx2)^+$] that originate neuroblasts [type A cells; $GFAP^-$/ $Dlx2^+$/ doublecortin $(Dcx)^+$] (Fig. I.3 C) (Riquelme *et al*., 2008). Neuroblasts migrate along the rostral migratory stream to the olfactory bulb, where they differentiate into granule cells and interneurons (Tavazoie *et al*., 2008; Zhao *et al*., 2008).

The SGZ is a brain region composed by radial glia-like cells also designated by type I cells, which express nestin, GFAP and Sox2 [SRY (sex determining region Y) – box2]. These cells proliferate and generate type II cells which differentiate to granule neurons. Type I and II cells are thus considered the neural progenitor cells of the SGZ (Siebzehnrubl *et al*., 2011; Zhao *et al*., 2008) (Fig. I.3 E/F).

In summary, adult NSC are not a population of fully undifferentiated cells; instead, they are a subset of cells that exhibit common features with differentiated astrocytes, such as the expression of GFAP but, concomitantly, they also exhibit certain RGC properties. Adult NSC are present specifically in the SVZ and SGZ brain regions (Doetsch, 2003; Duan *et al*., 2008). Following the evolutionary process during brain embryo development, such as the one described above, RGC are the *in vivo* primary precursors of neurons and glia, and postnatally, radial glia transition into astrocytes persists through type B cells (SVZ) and type I cells (SGZ) niches (Merkle *et al*., 2007; Riquelme *et al*., 2008). In addition to their role as stem cells, within adult neurogenic niches, these astrocyte-like cells work as sensors and regulators of the microenvironment. They envelop and contact all cell types and structures in the niches, including blood vessels and the basal lamina, allowing them to integrate diverse signals from many sources. Moreover, these cells are commonly associated via gap junctions and they have the ability to form a syncytium, which may allow them to propagate signals locally or throughout the entire niche, controlling activation and differentiation of stem cells (Riquelme *et al*., 2008).

To understand the generation of neurons from cells with astrocytic properties, it is necessary to refer some signalling pathways. SVZ astrocytes are adjacent to the ependymal cell layer expressing the protein Noggin that may promote SVZ neurogenesis by antagonizing signalling of the bone morphogenetic proteins (BMP). In SGZ neurogenesis, hippocampal astrocytes promote the differentiation of adult hippocampal progenitor cells into immature neurons. Lie and al. (2005), proved that the blockade of the Wnt signalling pathway inhibit the neurogenic activity of astrocytes *in vitro* and SGZ neurogenesis *in vivo*, suggesting that hippocampal astrocytes may act through Wnt signalling (Lie *et al*., 2005; Zhao *et al*., 2008).

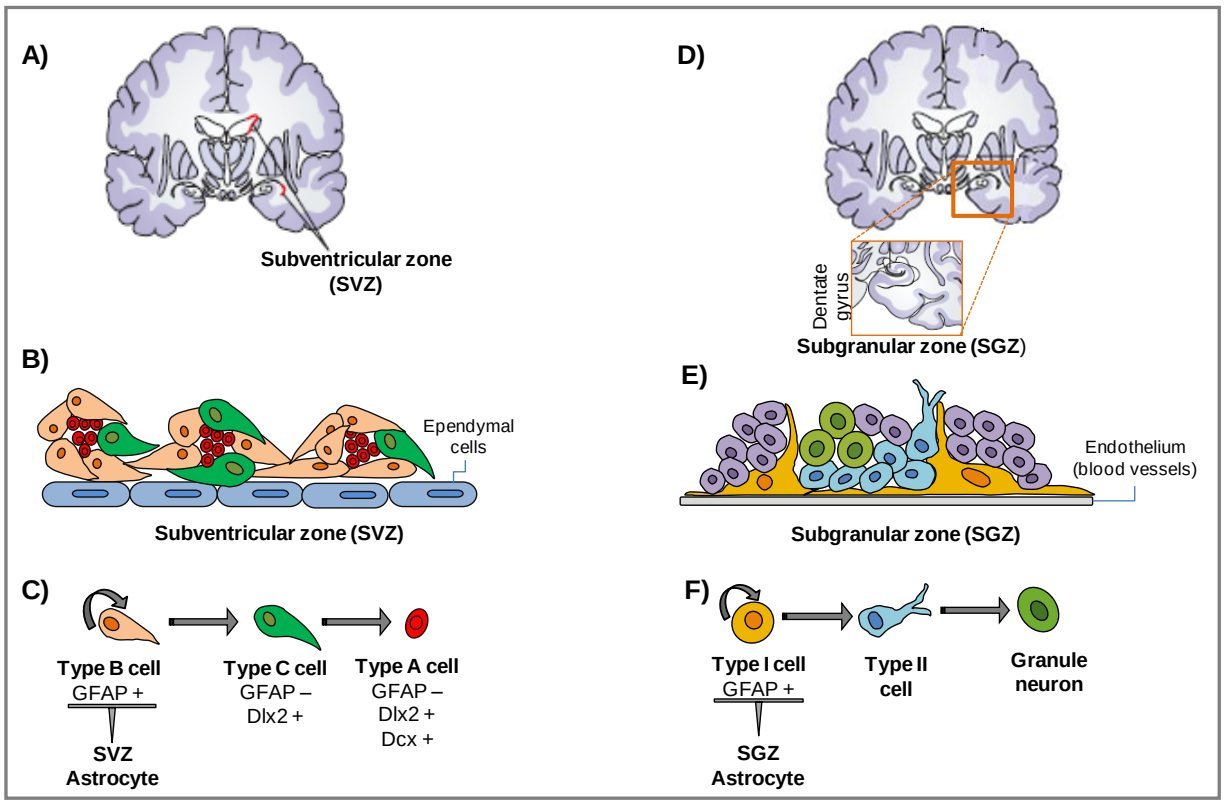


Fig. I.3 – The adult niches, cell types and stem cell lineage. A) Frontal schematic representation of the adult mouse brain showing the location of the subventricular zone (SVZ). B) Schematic representation showing the cell types and their organization in the SVZ. Ependymal cells (blue dark) line the lateral ventricle. Groups of neuroblasts (red) travel through tunnels formed by the processes of SVZ astrocytes (salmon). Focal clusters of rapidly dividing Type C cells (green light) are scattered along the network of chains of neuroblasts. SVZ astrocytes occasionally extend a process to contact the lateral ventricle. C) SVZ astrocytes (Type B cell) act as stem cells in this region and divide to generate transit-amplifying (Type C cell), which in turn divide to generate the neuroblasts (Type A cell) that migrate to the olfactory bulb. D) Frontal schematic representation showing the location of the SGZ in the hippocampus. The SGZ lies between the granular cell layer and the hilus. E) Schematic representation showing the cell types and their organization in the SGZ. SGZ astrocytes (Type I cell, orange) are in close proximity to blood vessels (endothelium, grey). Endothelial cells are likely an important source of signals for neurogenesis. F) SGZ astrocytes divide to generate intermediate precursors (Type II cell, blue), which generate granule neurons (green dark). Dcx (doublecortin), Dlx2 (distal-less homeobox 2), GFAP (glial fibrillary acidic protein). Adapted from Doetsch (2003); Riquelme *et al.* (2008); Vescovi *et al.* (2006).

1.3. Therapeutic potential of NSC

The physiological loss of tissue homeostasis during life gives rise to a progressive and extensive decline in the physical and cognitive performance. This loss could be aggravated by pathological factors triggering the development of disorders such as PD, AD and ALS. Apart from neurodegenerative disorders, the brain tumours are another serious pathology that dramatically affects the quality of life and life expectancy in patients (Artegiani and Calegari, 2012; Goldman and Windrem, 2006).

For many CNS diseases, particularly in cancer, treatment options are very limited. Surgical intervention is restricted by the local limited accessibility, as well as by the high risk of disturbing vital normal brain functions. Also, the use of systemic chemotherapeutics might not be effective due to the

largely impermeable blood-brain barrier (BBB) (Joo *et al*., 2012). Similarly to the chemotherapy, radiation therapy is a common modality for the treatment of various brain tumours; however it is clear that these therapeutic regimens may themselves produce injury, being often associated with significant cognitive impairment (Joo *et al*., 2012; Noble, 2000). Hence, stem cell-based therapies have been lately proposed and might represent a plausible alternative strategy in several of these and other disorders (Bonnamain *et al*., 2012; Pluchino *et al*., 2005). NSC display a strong tropism for tissue lesions and seem to migrate towards critical sites to release molecules aimed at preventing cell death and facilitating regeneration of targeted cell populations. Moreover, this migratory capacity makes these cells of particular interest as therapeutic delivery vehicles directly into the lessoned area using genetically engineered stem cells (Bonnamain *et al*., 2012; Noble, 2000). Thus, NSC therapeutic potential can be addressed in two different ways - through endogenous NSC or, in the other hand, through the transplantation of these cells, depending on the type of brain injury (Pluchino *et al*., 2005). Endogenous adult NSC, existing mainly in neurogenic niches (namely SVZ and SGZ, as described in the previous section) may endure neurogenesis and gliogenesis in response to several different injuries such as those occurring during inflammatory, ischemic, or traumatic events, acting as part of an "intrinsic" brain self-repair process during adulthood (Pluchino *et al*., 2005; Taupin, 2006). It is believed that these insults may trigger a cascade of cellular and molecular signals, mediated by the release of soluble mediators (cytokines, chemokines, metalloproteases, adhesion molecules, etc) capable of supporting neurogenesis and gliogenesis that, in turn, favours brain regeneration (Chang *et al*., 2012; Pluchino *et al*., 2005). Nevertheless, the intrinsic signals are not sufficient to promote proliferation and differentiation of NSC. Therefore, regeneration could be triggered by the stimulation of endogenous repair mechanisms at sites of degeneration leading NSC to secrete a plethora of trophic factors able to protect and prevent neural cell damage, and to re-establish the functional interactions between neural and glial cells (De Feo *et al*., 2012; Taupin, 2006). Studies revealed that in the SVZ, newly generated neuronal cells migrate partially through the rostro-migratory stream to the sites of nerve cell degeneration (Arvidsson *et al*., 2002). For instance, recent studies report that selective-serotonin reuptake inhibitors, such as fluoxetine, stimulate proliferation of NSC and increase the number of cells with neuronal features. It was shown that fluoxetine promotes both proliferation and neuronal differentiation of NSC and exerts protective effects in NSC, suggesting its therapeutic usage in several neurodegenerative diseases, such as AD and PD, considering its actions on NSC (Chang *et al*., 2012). Despite the generation of new neuronal cells at the sites of degeneration, this is insufficient to promote functional recovery after neurological injuries. This failure results from the low number of new neurons generated, or even because they are non-functional (Joo *et al*., 2012; Taupin, 2006).

The transplantation of NSC appears as another type of cellular therapy based mainly on the ability of transplanted NPC to migrate and adapt their behaviour and fate to the CNS microenvironment, and to promote neuroprotection via different and articulated strategies encompassing not only cell replacement, but also the so-called "bystander" effect (a mode of action named "therapeutic plasticity") (Colleoni and Torrente, 2008; De Feo *et al*., 2012; Pluchino *et al*., 2005). Anatomic and pathologic characteristics of CNS disorders such as PD, spinal cord injury,

Huntington´s disease or stroke, suggest that intracerebral transplantation of these cells directly at the site of the lesion would be the more appropriated strategy to facilitate tissue regeneration (Bonnamain *et al*., 2012). Neural stem/progenitor cells (NSPC) can spontaneously differentiate *in vivo* under the influence of the microenvironment, in cells with the desired phenotypes. In fact, it was recently shown that undifferentiated human NSPC have the capability to survive and differentiate into neurons and glial cells after xenotransplantation in the rat spinal cord (Mothe *et al*., 2011). On the other hand, this therapeutic strategy has also been considered for tumour treatment because the behaviour of NSC although capable of being influenced by the tumour signals, have the ability to target the primary tumour mass, tumour outgrowths and distant tumour pockets (Colleoni and Torrente, 2008; De Feo *et al*., 2012). The transplanted NSC might carry some genes/factors of interest, such as genes encoding proteins that induce differentiation of neoplastic cells and/or their signal-transduction mediators, cell cycle modulators, apoptosis-promoting agents, anti-angiogenesis factors, immune-enhancing agents and oncolytic factors (Colleoni and Torrente, 2008). The signals and factors that might influence the tumour tropism of NSC and their interaction within the tumour environment are currently under investigation. However, it is speculated that soluble factors (overexpressed by tumour cells) may be an important signal for the long-range attraction of NSC from distant sites. Recent studies indicate that tumour-upregulated vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1) act as soluble chemotactic factors inducing tropism of NSC (mainly to glioma). The presence of these chemoattractant factors may allow NSC to communicate with each other and facilitate the observed migration (Colleoni and Torrente, 2008; Joo *et al*., 2012). Particularly in brain tumours, over the last years, studies about the potential use of NSC as a therapeutic tool for glioblastoma have been reported. For instance, in 2000, Benedetti *et al.* reported that NSC genetically modified to produce interleukin-4 (IL-4) that could promote tumour regression and prolonged survival in mice injected intracranially with the GL261 mouse glioma cell line (Benedetti *et al*., 2000). Later, Ehtesham *et al.* (2002) demonstrate that the inoculation of NSC transduced with tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (a member of the tumour necrosis factor protein super family) into human glioma drove to potent induction of tumour cell apoptosis and consequently to a highly significant decrease in tumour volume.

Nowadays, due to the limited delivery of drugs to brain tumours (hindered primarily by BBB), drug delivery nanosystems are being studied, such as nanoparticles (NP), which include nanospheres and nanocapsules, micelles, dendrimers, nanocrystals, and nanogolds (Roger *et al*., 2011). The therapeutic agents (such as anticancer drugs), can be encapsulated into the NP (Lopes *et al*., 2011) and then carried by them (Garcion *et al*., 2006; Huynh *et al*., 2009; Roger *et al*., 2011). Thus, NP can protect therapeutic agents from chemical or enzymatic degradation and allow their sustained and controlled release (Roger *et al*., 2011), being these drug-loaded NP administered by systemic or direct delivery to the CNS. Thus, due to the special tropism of NSC, NP and stem cells to glioma cells, these are promising tools to treat brain tumours (Ferreira *et al*., 2008). NP have been shown to enter inside the target cells *via* passive transporter (Banerji and Hayes, 2007) or active endocytosis (Lorenz *et al*., 2006; Rejman *et al*., 2004). Once inside the cells, NP are usually transported to the endo-lysosomal system, where they are destroyed, and to release the pharmacotherapeutic agents in the cytoplasm.

Inside NSC, NP can be administered directly into the brain *via* a intratumoural or contralateral injection into the tumour mass (Fig. I.4) (Roger *et al*., 2011).

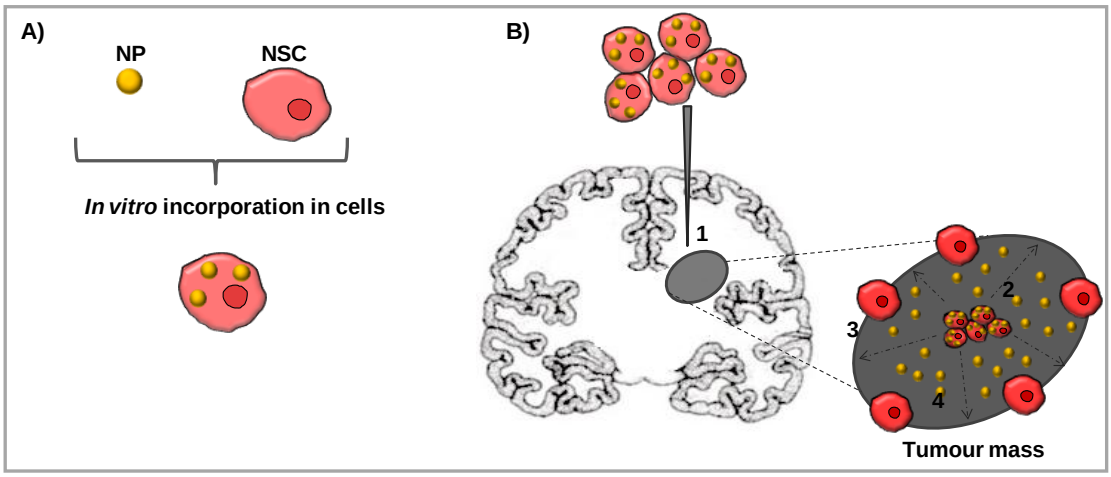


Fig. I.4 – Schematic representation of nanoparticles delivery by neural stem cells inside a brain tumour. A) Incorporation of nanoparticles (NP) containing anticancer drugs into neural stem cells (NSC). B) Delivery strategy: 1. Intratumoural injection of NSC loaded with NP into the tumour mass. 2. Migration of NSC loaded with NP. 3. NSC distribution at the border between normal brain parenchyma and the tumour mass. 4. Release of the therapeutic agent. Adapted from Roger *et al.* (2011).

The stimulation of endogenous neural progenitor and stem cells, and the transplantation of adult-derived neural progenitor and stem cells may represent valid strategies for the treatment of a broad range of CNS diseases and injuries. However, their clinical application is limited by both ethical and logistical problems such as their isolation and their immunological compatibility in allogenic transplantation (Colleoni and Torrente, 2008; Taupin, 2006).

1.4. *In vitro* culture of NSC: the neurosphere assay

NSC sources are located in inaccessible areas within the brain, thus severely limiting their clinical utility. Hence, it is highly desirable and urgent to find an alternate source of neural cells (Sun *et al*., 2011; Suzuki *et al*., 2004). Working on an attempt to find a method of expanding NSPC, several groups contributed to the discovery of two culture systems for the maintenance and expansion of NSC: neurospheres (NS, in suspension) and adhesive substrate-bound (adherent) cultures. Both systems are good culture methods; however, although the adherent culture is richer in NSC and grow significantly faster than NS, it could only maintain robust growth during 6-7 passages, while NS could be maintained for more than 10 passages (Sun *et al*., 2011).

"NS", the culture system used in the present thesis, was performed for the first time by Reynolds and Weiss (1992). They have demonstrated that cells from embryonic or adult nervous system can be cultivated and propagated *in vitro* as NS from single NSC, suggesting their potency for self-renewal (Zhao *et al*., 2008). NS agglomerate and form spherical clusters and can be expanded in long term suspension cultures, in the presence of growth factors (Bez *et al*., 2003; Breier *et al*., 2010). These cellular structures represent three dimensional heterogeneous clusters of proliferating cells,

including stem cells, committed progenitors, and differentiated cells such as neurons, astrocytes and oligodendrocytes (Bonnamain *et al*., 2012; Breier *et al*., 2010; Garbossa *et al*., 2012).

The morphological and functional heterogeneity of these free-floating structures is observed and evaluated through several factors as size, apoptosis, phagocytosis, proliferation/self-renewal, differentiation and migration. Spheres of different sizes could be generated from cells plated at the same time and under identical culture conditions, resulting in larger NS (with a dark core) and/or smaller ones (more translucent) (Bez *et al*., 2003). The size of NS influences their cell activity in that the highest cell activity (i.e., mitosis, protein synthesis) occurs at the periphery of the agglomerates more accessible to nutrients and oxygen, while necrosis, and low or absent mitotic and transcriptional activity, are typical of the inner layers where nutritional exchanges are more difficult. Moreover, results of propidium iodide (PI)/Hoechst staining (to detect cellular death), showed some PI^+ cells on the core of the big floating-free structures (Fig. I.5A) (Bez *et al*., 2003; Sun *et al*., 2011).

NSC derived from NS are not synchronized and can be in any phase of the cell cycle. Besides nestin (Fig. I.5C1) and Sox2 (Fig. I.5D1), markers of NPC, NS also express MAP2 (microtubule-associated protein 2, mature neuron marker) and GFAP, showing that the culture is also composed of mature neural populations (Sun *et al*., 2011). As expected, $nestin^+$ undifferentiated cells are located in the periphery of the sphere, while differentiated neuronal (βIII-tubulin+) and glial (GFAP+) cells (Fig.I.5B) reside in the center, probably due to a growth factor gradient from the outside to the inside of the sphere (Breier *et al*., 2010). Cell proliferation might be the most important feature of NS, and both the epidermal and fibroblast growth factors (EGF and FGF, respectively) have a crucial role in the NSPCs cell cycling maintenance. The self-renewal and proliferation can be shown and quantified by the presence of immature cells (nestin+) and cycling cells [5-Bromo-2'-Deoxyiridine (BrdU+)] in the outer layer of the cluster. This double immnunofluorescence staining shows that $BrdU^+$ cells are also $nestin^+$ cells (Fig. I.5E4) (Sun *et al*., 2011). In the same way that growth factors maintain the proliferative characteristics, their withdrawal drive cells to stop proliferating and start differentiating and expressing neurotrophins. This, will cause a 50% decrease in BrdU labelling and nestin expression, and an increase in the number of $GFAP^+$ and β-tubulin III^+ cells (Schwindt *et al*., 2009). With growth factors withdrawal, neural progenitor cells migrate radially out of the sphere onto a given extracellular matrix (ECM), and thereby differentiate into cells expressing neural and glial markers which form the migration area, while the zone pattern inside the sphere disappears. After some time of differentiation, $βIII\text{-tubulin}^+$ cells are found at the edge of the sphere while $nestin^+$ and $GFAP^+$ cells are heterogeneously distributed throughout the sphere (Breier *et al*., 2010; Schwindt *et al*., 2009). Additionally, the dissociation of NS also favours differentiation of dissociated cells into $O4^+$ oligodendrocytes, $GFAP^+$ astrocytes or $βIII\text{-tubulin (TuJ1)}^+$ neurons (Bez *et al*., 2003; Sun *et al*., 2011).

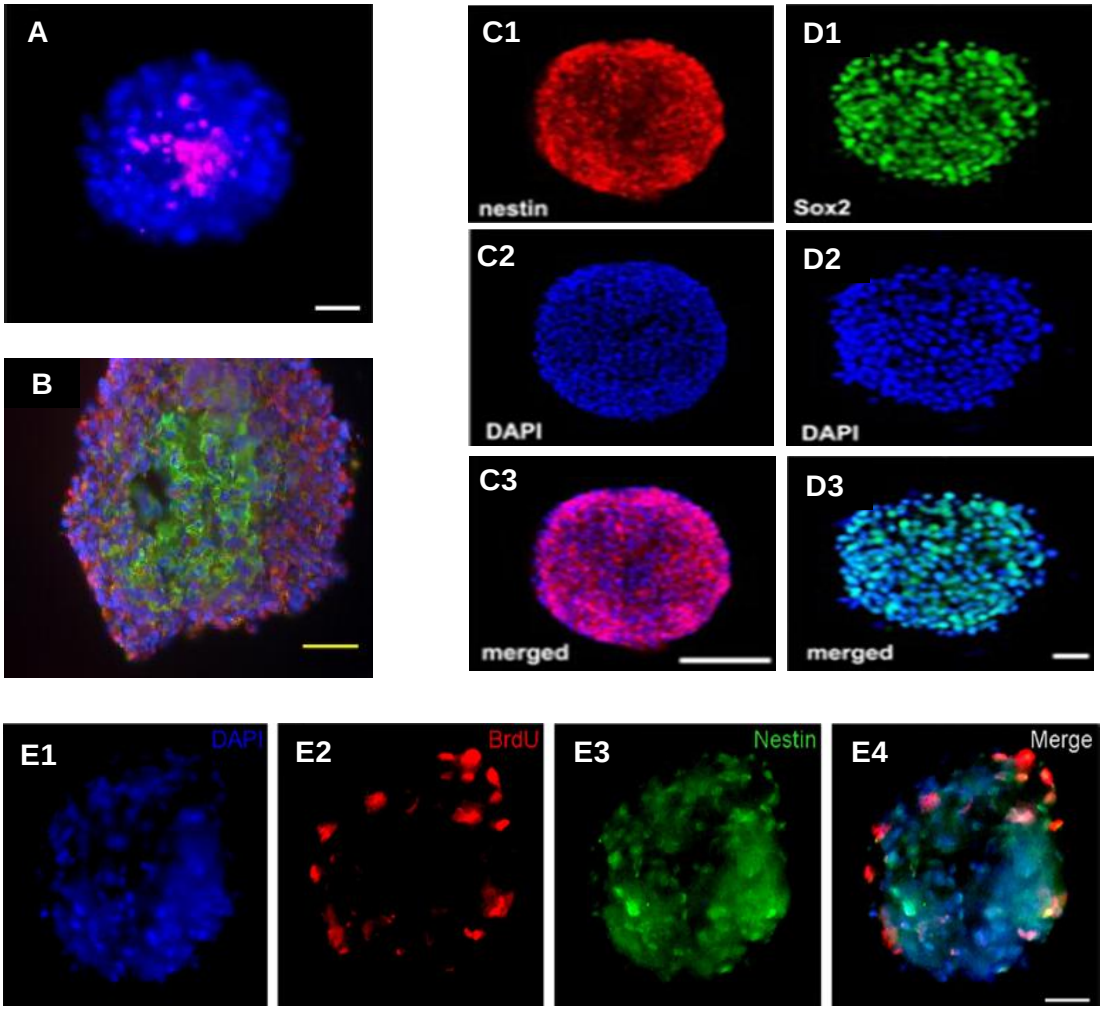


Fig. I.5 – Principal feactures and phenotypes of neurospheres. A) Dead cells (red) in the core of the neurosphere (NS) are labeled with propidium iodide (PI) (red) and nuclei are counterstained with Hoechst 33342 (blue). Scale bar represents 30 µm. B) NS with a central core of glial fibrillary acidic protein (GFAP) positive glia (green) surrounded by nestin (red) positive neural stem cells at the sphere edge. Scale bar represents 50 µm. C1) and D1) NS contain nestin-positive (red) and Sox2-positive [SRY (sex determining region Y)-box 2 (green)] cells, respectively. C2) and D2) Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, blue). C3) and D3) merge of images C1+C2 and D1+D2, respectively. Scale bar represents 100 µm in the series C and 20 µm in the series D. E1-4) Staining for nuclei (DAPI), proliferation (BrdU, red) and nestin (green). Scale bar represents 30 µm. Figure A and E are from Sun *et al.* (2011), B is from Lu *et al.* (2010), and C and D from Wang *et al.* (2007).

As referred above, the characteristic migration of these cluster cells is easily assessed because upon growth factors withdrawal, NPC start leaving the sphere in a 90^{o} angle and their travel distance over time can be measured through a phase contrast microscope. The process of migration is regulated by intracellular, as well as extracellular stimuli. The migration of human NPC out of the neurosphere is controlled by the mitogen MAPK (mitogen activated protein kinase) ERK1/2 (Extracellular signal-regulated protein kinases 1 and 2) –dependent and –independent pathways. Moreover, human neural progenitor cell migration is preserved on collagen, fibronectin and poly-L lysine matrices, indicating a crucial role of the ECM in neural migration not only *in vivo*, but also *in vitro* (Breier *et al*., 2010).

Overall, the importance of having a reliable model of NSPC is related to the fact that these cells can serve as a valuable source of cell type-specific somatic precursors for neural transplantation, thus

offering a potential starting point for therapy of neurodegenerative diseases. In addition, this model can also be a useful tool for testing neurotoxic substances for their abilities to interfere with basic process of brain development, such as proliferation, migration, differentiation and apoptosis, a procedure that was called by developmental neurotoxicity testing (Breier *et al*., 2010).

2. Neural stem cells, tumour stem cells and brain tumours: dangerous relationships?

Brain tumours are a wide group of abnormal masses of tissue as a result of uncoordinated proliferation of cells (neoplasms) with a high incidence in children and adults with the poorest outcome among the human cancers. In adults, their incidence is relatively high, especially in elderly people; in children, brain tumours are the second commonest type of cancer (17% of all childhood's cancer) and cause 25% of cancer deaths. Until now, only small changes have been registered in these numbers and they continue to be associated with very high morbidity and mortality (Dirks, 2008; Sutter *et al*., 2007).

There are different types of brain tumours with neuroepithelial origin, that have been classified based on the histological resemblance of tumour cells to cells present in the adult brain, and thus focusing in the cell types that compose the tumour mass (Sanai *et al*., 2005) (Fig. I.6). Although this classical classification is used by most authors, more recently, and according to the 2007 report published by the World Health Organization (WHO), brain tumours have been classified not only based on a single cell type but there were included also mixed type of tumours, such as the mixed neuronal-glial (Louis *et al*., 2007; Sutter *et al*., 2007; Vescovi *et al*., 2006).

Brain tumours are a heterogenous group of malignancies that originate and reside within the brain, contrary to metastatic brain tumours that originate from a primary cancer outside the CNS and spread to the brain (Germano *et al*., 2010). They are organized as a cellular and functional hierarchy based on a subpopulation of brain tumour cells that have, surprisingly, stem cell properties. Both tumour and NSC are morphologically simple, express many of the same specific genes and proteins, can differentiate into cells of various shapes and sizes, and have the ability for extensive growth (Wechsler-Reya and Scott, 2001). These shared features, together with the ability of this tumour cell population to form NS and to be repeatedly passaged/proliferated (self-renewal capacity), are used as indicators of stem cell properties of the tumour cells, giving them the designation of brain cancer stem cells (CSC). However, although CSC and NSC shared a lot of characteristics, there are some differences between them, particularly in what concerns to cell proliferation. In fact, while NSC possess a tight control of the asymmetric cell division and can maintain a relative balance between self-renewal and differentiation, CSC are characterized by uncontrolled proliferation or self-renewal. Hence, it is thought that if NSC acquire genetic mutations that impair asymmetric divisions, they might turn into CSC and induce tumourigenesis (Dirks, 2008; Vescovi *et al*., 2006; Yao *et al*., 2009).

Current treatment strategies for brain tumours include surgery, radiotherapy, and chemotherapy. Nevertheless, these clinical interventions modestly improve patient survival. In addition, treated patients often have intellectual impairment related to chemotherapy and radiotherapy. Moreover, even brain tumours classified as benign can be lethal due to their location in surgically inaccessible areas (Yao *et al*., 2009; Dirks, 2008). The core of treatment failure derives from the poor understanding of the cellular and molecular mechanisms regulating tumour growth. Thus, it is very important to understand how cells in the diverse tumour populations initiate and maintain growth, and which are the molecular mechanisms involved and the brain cells that suffer malignancy transformation and give rise to brain tumour (Dirks, 2008). Hence, it is necessary to analyse asymmetric and/or symmetric division directly on tumour stem cells and to compare the signalling pathways involved in NSC with those in CSC proliferation (Yao *et al*., 2009).

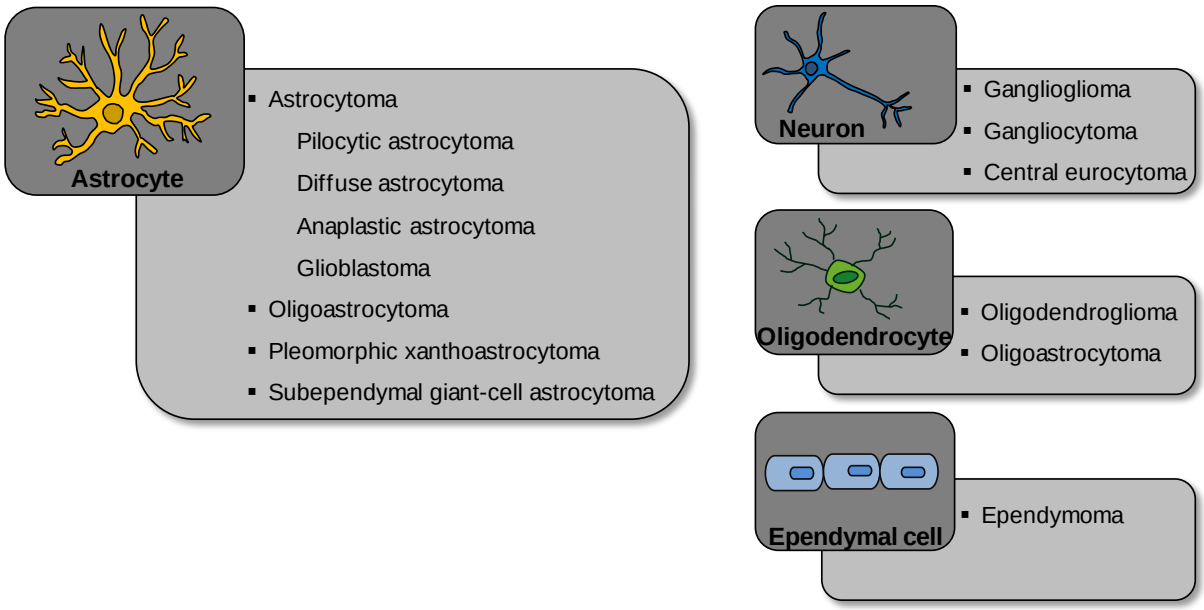


Fig. I.6 – Cell types and associated tumours of the central nervous system. Brain tumours have been mostly classified based on the histological resemblance of tumour cells to central nervous system (CNS) cells, such as astrocytes, neurons, oligodendrocytes and ependymal cells. Adapted from Sanai *et al.* (2005).

2.1. Gliomas – an overview

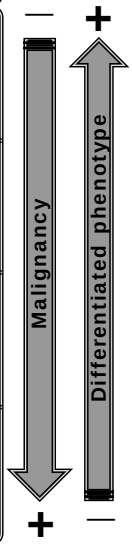
Gliomas are the most common type of brain tumours, accounting for more than 70%. Gliomas consist of a heterogenous mixture of several glial phenotypes, composed by immature cell types, poorly differentiated neoplastic astrocytes and mature cells. These tumours can either develop by dedifferentiation from a low grade tumour ("secondary glioma") or can arise "de novo" ("primary glioma"). Differences in clinical and molecular features of the two types of glioma point to a distinct pathogenesis (Park and Rich, 2009; Siebzehnrubl *et al*., 2011). Some controversy still exists in turn of the origin of these brain tumours, once some authors refer to NSC as the cells that undergo molecular and cellular transformation generating the tumoural growth, while others think that glioma arises from

dedifferentiation of mature glia (Sanai *et al*., 2005; Vescovi *et al*., 2006). However, if the last hypothesis has been increasingly discarded, the glioma origin from NSC is the most accepted and the one intensively researched reason why this assumption was followed in the present thesis.

According to their histological features, there are three main types of the most common gliomas: astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas (Siebzehnrubl *et al*., 2011). According to the WHO, gliomas are organized into four different grades based on histological properties of cellular composition, nuclear morphology and atypical cell stage, mitotic activity, necrotic features, and microvascular proliferation (Louis *et al*., 2007; Ohgaki and Kleihues, 2005). A higher histological grade corresponds to a less differentiated phenotype and to an increasing malignancy (Park and Rich, 2009). Regarding astrocytomas, the main focus of the present work, they can be classified in low grade/WHO Grades I and II or high-grade or malignant/WHO Grades III and IV (Louis *et al*., 2007; Siebzehnrubl *et al*., 2011) (Table I.1.).

Table I. 1 – Astrocytoma grades. Astrocytomas can be divided in four groups based on their malignant grade and general tumour characteristics, in accordance to World Health Organization.

Grade	Tumour Type	Criteria
I	Pilocytic astrocytoma	▪ Low proliferating ▪ Discrete ▪ Non invasive tumour
II	Diffuse astrocytoma	▪ Modest proliferating ▪ Partly invasive tumour
III	Anaplastic astrocytoma	▪ Fast proliferating ▪ Invasive tumour
IV	Glioblastoma multiforme	▪ Rapidly proliferating ▪ Highly invasive tumour



The latter two tumour subtypes are considered the most malignant gliomas and are associated with a very poor prognosis. Particularly, glioblastoma multiforme (GBM) accounts for 50% of primary brain tumours and only 5% of patients have a 5 years survival rate, meaning that the average survival rate is approximately only 14 to 15 months (Ohgaki and Kleihues, 2005; Stupp *et al*., 2005; Sutter *et al*., 2007). The peak of the onset of GBM is between 50 and 55 years, which makes it an age-related disease, and male are slightly more prone to this pathology than female individuals. Moreover, the black people undergo an incidence 2-3 times higher than white people (DeAngelis, 2001; Stupp *et al*., 2005).

Although gliomas are a relatively rare form of cancer, they account for a disproportionately high morbidity and mortality because their location in the brain is generally problematic to surgery and other therapies. Their treatment is a daunting challenge to clinicians due mainly to the lack of effective

therapeutic options. Gliomas rarely metastasize outside of the brain, but instead, infiltrate extensively into surrounding normal brain tissue. Thus, although the surgery is not curative it can establish the diagnosis. It is taken as the first choice because the resection of the tumour is important to decrease the pressure it exerts and is usually followed by focal external beam radiation (Germano *et al*., 2010; Park and Rich, 2009). Subsequently, radiation therapy and chemotherapy increase survival, but disease recurrence is virtually inevitable (Park and Rich, 2009). Besides these difficulties it is observed that the average survival after surgical resection alone is six months with only 7.5% of patients surviving two years post-operatively. Adding radiation therapy, this average survival prolongs to nine months, while systemic chemotherapy provides minimal survival benefits (Siebzehnrubl *et al*., 2011).

The latest researches have attempted the use of systemic chemotherapy with alkylating agents such as temozolomide (TMZ). Thus, since 2005, the care standard for newly diagnosed patients with GBM includes resection, fractionated radiation concurrent with TMZ chemotherapy, followed by TMZ alone (DeAngelis, 2001). This association between surgery, radiation and chemotherapy is not able to avoid that the recurrence in high-grade gliomas will occur in more than 90% of cases, frequently within 2 cm of the original site, but 10 to 20% of the cases may develop new lesions at distant sites (Germano *et al*., 2010). However, it showed to increase the overall survival by 2.5 months, the progression-free survival by two months and the two-year survival by 16% (DeAngelis, 2001). Despite the important effort that has been made to find therapeutic agents and the development of GMB models representing the features of human malignancy, the poor identification of the malignant tumour initiating cells has limited the development of more effective therapies (Siebzehnrubl *et al*., 2011).

Overall, the lack of knowledge about the relationship between the normal cell and the tumourigenic cellular transformation, as well as the poor information about the characteristic tumour heterogeneity, have delayed the identification of new molecular targets and the development of novel target therapeutic processes to apply in GBM treatment. Hence, it is necessary a better understanding of the mechanisms involved in the tumour origin and progression, to allow the discovery of new and more effective therapies.

2.2. Cancer stem cell hypothesis

Tumours are currently viewed as a disruption of the cellular organization mechanisms resulting from accumulation of genetic and epigenetic events at the germ line and somatic levels. The cell of origin of cancer has been a strongly debated topic throughout the history of cancer research, and over the last few years the idea that cancer is a disease driven by CSC has emerged (Bapat, 2007; Houghton *et al*., 2007), as referred on the beginning of this chapter.

Although the concept of CSC has been originally proposed in 1990s, the first evidence of the presence of cells with stem-like characteristics in human brain tumours was only reported later by Ignatova *et al*. (2002) who isolated clonogenic neurosphere-forming precursors from post-surgery specimens of human GBM. These stem-like cells expressed both neuronal and astroglial markers, together with several key determinants of neural stem-cell fate (Fig. I.7). Afterwards, several groups

have shown the presence of these cells within brain tumours; for instance, two different groups demonstrated that both GBM and medulloblastoma contain NS-forming cells that can give rise to neuronal and astroglial-like cells (Hemmati *et al*., 2003; Singh *et al*., 2003).

The identification of CD133 (prominin 1) immunoreactive cells in brain tumours and their characterization as CSC was based on the fact that the glycosylated epitope of the CD antigen AC133 appeared to be restricted to stem cells. These CD133+ cells in brain tumours were shown to be highly tumourigenic (Germano *et al*., 2010) and express molecular markers associated with neural precursors, such as nestin, the transcription factor Sox2, the RNA binding protein Musashi, B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1), Notch, the transcription factor Emx2, paired box 6 (Pax6) and the ligand for the receptor notch 1 (Jagged1). However, when exposed to differentiation conditions, these cells downregulate the expression of these immature markers and acquire immunoreactivity for βIII-tubulin (for neurons), GFAP (for astrocytes) and platelet-derived growth factor receptor-α (PDGFRα) (for oligodendrocytes) (Dirks, 2008; Germano *et al*., 2010; Sutter *et al*., 2007). Additionally, the CD133+ population is resistant to several chemotherapeutic agents (such as TMZ, carboplatin, placlitaxel and etoposide) and to radiation therapy (Germano *et al*., 2010; Yao *et al*., 2009). These cells by showing karyotypic and other genetic alterations are indeed neoplastic and not residual NSC entrapped in the tumour (Fig. I.7) (Germano *et al*., 2010).

Altogether, these findings demonstrate that: a) different brain tumours are composed by transformed, undifferentiated neural precursors that respond to the same mitogens and activate adult NSC; b) tumour stem-like cells possess some of the molecular features of NSC; c) CD133, a cell-surface protein that is a marker of normal human neural precursors, can be used for the enrichment of tumour stem-like cells from brain tumours; and d) tumour stem-like cells have the ability to maintain stem cell function and to promote tumour growth. These findings and assumptions led to the cancer stem cell hypothesis in GBM and in other solid tumours, such as medulloblastomas and ependymomas (Taylor *et al*., 2005).

In the core of the relation between NSC and CSC there is a difference related to self-renewal. While NSC can maintain a controlled balance between self-renewal and differentiation, CSC are more long-lived. Indeed, these tumour-derived NS could be cultured for at least four months without relevant changes in their proliferative properties, whereas normal NS grown under identical conditions persisted no longer than one month in culture (Sutter *et al*., 2007). Hence, this uncontrolled proliferation can be in the origin of the brain tumour. So, if a normal NSC, during its life, accumulates enough mutations and undergoes neoplastic transformation, it would then become a CSC, forming and maintaining a brain tumour. Moreover, a recent work developed by Jackson and colleagues (2006), has shown that NSC into the SVZ express PDGFRα and the administration of exogenous PDGF (platelet-derived growth factor) initiates a signalling cascade that induces aberrant proliferation of these cells leading to formation of hyperplastic lesions, with properties like gliomas, which, however, regress upon PDGF withdrawal. So, this deregulation of the PDGF signalling suggests that genetic or epigenetic mechanisms affecting the proliferation of NSC can be an early factor in brain tumour formation (Sutter *et al*., 2007). Thus, it is hypothesized that CSC can be generated from the oncogenic transformation of NSC residing in the SVZ, rather than from lineage of committed progenitors (Fig.

I.7). In fact, there is another hypothesis for the origin of CSC that refers to the processes of dedifferentiation from astrocytes (Germano *et al*., 2010), which will not be any further discussed along the work.

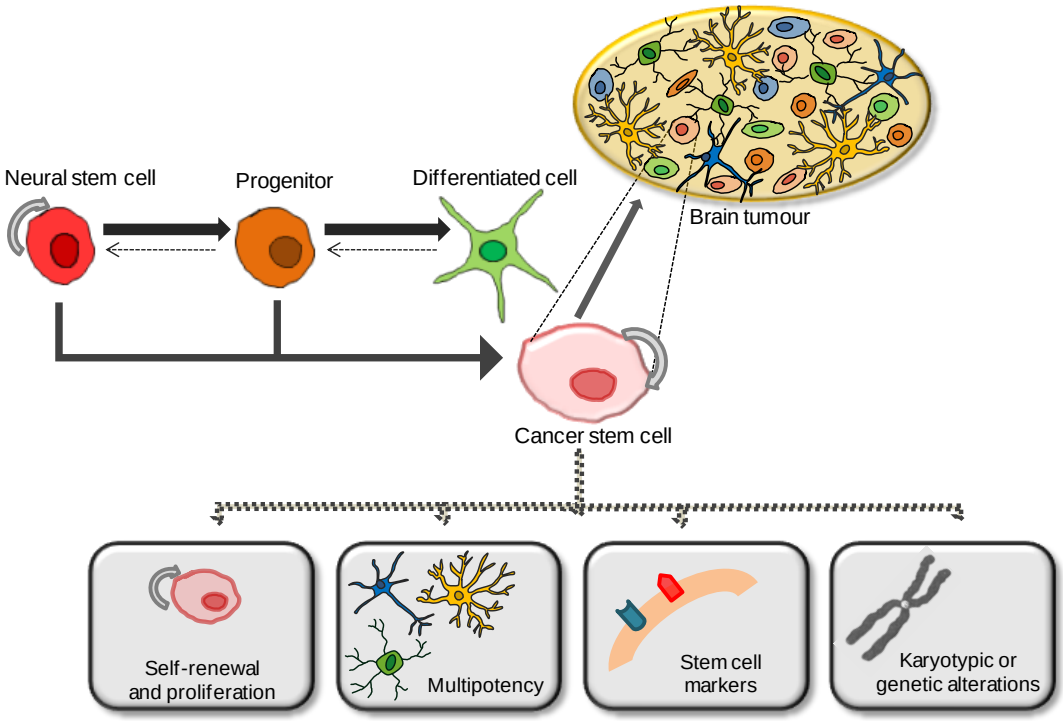


Fig. I.7 – Schematic representation of the relationship between neural stem cells, neural progenitor cells, cancer stem cells and brain tumours. Cancer stem cells (CSC) originate from neural stem cells (NSC) or progenitor/differentiated cells upon acquisition of genetic mutations, giving rise to brain tumours. The features of a brain tumour stem cell are: self-renewal, proliferation *in vitro*, multipotency with aberrant differentiation pattern, expression of stem cell markers and karyotypic or genetic alterations. Adapted from Sutter *et al.* (2007).

Because of brain tumours complex organization, its effective treatment is a very difficult task. The conventional therapies that act over dividing cells, although reduce partially tumour bulk, they often do not prevent tumour regrowth, probably because this kind of approach is not able to destroy the CSC (Sutter *et al*., 2007). This resistance of CSC to radiation and chemotherapy has been attributed to the quiescent phenotype and enhanced DNA (deoxyribonucleic acid) repair in CSC, as well as to the expression of drug efflux pumps and anti-apoptotic proteins (Gilbert and Ross, 2009; Houghton *et al*., 2007). The work developed by some researchers, as Bao *et al*. (2006) have shown that $CD133^{+}$ glioma cells survive to radiation and chemotherapy due to the activation of DNA damage response, leading to a better repair of radiation-induced DNA damage, as well as by the upregulation of drug resistance genes expression that make the CSC insensitive to chemotherapy (Sutter *et al*., 2007). This multidrug resistance phenomenon can be mediated by several ATP-binding cassette (ABC) transporters, such as the protein encoded by the multidrug resistance protein (MDR), the multidrug resistance-associated protein (MRP), and the breast cancer resistant protein (BCRP). Surprisingly, some of these transporters are also expressed in many kinds of normal stem cells (Kondo, 2006; Sutter *et al*., 2007). Moreover, Ghods *et al*. (2007) described the upregulation of anti-

apoptosis-related genes that may also account for treatment resistance (Sutter *et al*., 2007). Therefore, the discovery of new targets in CSC and more directed brain tumour therapies are new and promising concepts considered to be necessary to improve the actual poor prognosis of these highly aggressive and devastating tumours.

2.3. Cues for glioma origin

The determination of the cellular origin of gliomas is very important to better understand this disease and to develop new therapeutic approaches. As described above, it is thought that gliomas are originated by the transformation of NSC or NPC. According to some recent works, modifications in the normal glial NPC differentiation can lead to the generation of abnormal cells, instead of mature astrocytes (or oligodendrocytes). This hypothesis for glioma origin has been explored because NSC and NPC, share some characteristics with gliomas, such as: high proliferation and motility, association with blood vessels and white-matter tracts, evidence immature antigenic phenotypes, and "developmental" signalling pathways activation (Sanai *et al*., 2005). In addition, regarding tumour location, many gliomas are localized in the SVZ of the adult brain, a germinal region where NSC are also localized (Sanai *et al*., 2005), suggesting that gliomas can be generated by the cells of this region.

Proliferation is a very important feature of brain tumours, and in fact, the regions of the brain with a high degree of cellular proliferation are more sensitive to chemical or viral oncogenesis than areas with a low proportion of proliferating cells (Sanai *et al*., 2005). FGF and Notch signalling, that are involved in the regulation of NSC proliferation and renewal, are also involved in gliomas proliferation. Furthermore, epidermal growth factor receptor (EGFR) expression is up-regulated in primary GBM and in transiently dividing progenitors (type C cells) (Levy *et al.*, 2008).

Nowadays, the heterogeneity of glioma has been much studied because it has been observed that GBM have clonogenic NS i.e., forming cells that express both neuronal and glial markers upon differentiation (Ignatova *et al.*, 2002). However, the diversity of their progeny may be limited by the blocked-differentiation phenomenon, because although the transformed progenitor cells divide rapidly, their progeny are incapable of complete differentiation. Thus, the tumour phenotype may be defined by the direction and differentiation stage of the transformed progenitor population. For example, astrocytoma can arise from glial progenitors whose progeny can differentiate only along astrocytic lines. Although this scenario suggests that the cell of origin for gliomas is a stem cell, it also indicates that the cell of origin has undergone a transformation event that limits the normal differentiation of its progeny (Sanai *et al*., 2005). This heterogeneity of cell types in tumour mass is demonstrated by the identification of immature phenotype markers, such as Bmi-1, Nestin, Sox2, Musashi, stage-specific embryonic antigen-1/CD15 (SSEA-1), and activated Notch pathways, as well as by markers of mature astrocytes and oligodendrocytes (Park and Rich, 2009; Levy *et al.*, 2008). Hence, the state of differentiation is an important feature of the cell in the origin of gliomas because, although the influence of the state of differentiation depends on the oncogenes involved, the risk for malignant transformation of stem and progenitor cells in the CNS are at particularly also derives from their

activated cellular machinery (e.g., promitotic genes, telomerase activity, and antiapoptotic genes), which is determinant for tumour initiation, progression, or both (Park and Rich, 2009; Sanai *et al*., 2005). Other oncogenes are similarly involved in this transformation. For example, the combination of oncogene activation (RAS) and disruption of a tumour suppressor gene (Ink4a-Arf) provides an adequate oncogenic stimulation for tumourigenesis of both nestin-positive NPC and differentiated astrocytes (Uhrbom *et al.*, 2002). Moreover, astrocytic-specific inactivation of *NF1* gene fails to produce gliomas, but loss of this gene function in the development of the brain leads to the proliferation of glial progenitors and optic glioma formation. In addition, excision of two tumour suppressor genes, *p53* and *NF1* in mice results in the formation of astrocytomas only in the SVZ, a region made up by multipotent NSC (Park and Rich, 2009). Still, PTEN (phosphatase and tensin homolog), a tumour suppressor gene with an important function in the control of NSC proliferation and progenitor cells *in vitro* and *in vivo*, is inactivated in gliomas, thus allowing proliferation (Levy *et al.*, 2008).

The migration is other important concept related to gliomas. This migratory capacity of malignant gliomas represents a relevant challenge to any therapeutic strategy. In the injured adult brain, nestin-positive cells migrate to the site of injury from the SVZ, indicating that progenitor cells retain the ability to travel through mature parenchyma. This capacity for migration is an essential characteristic shared by gliomas and NSC. NSC and NPC appear to be associated with white-matter tracts and blood-vessel basement membranes and this is also true for gliomas. These findings suggest that NSC and tumour cells share a common substrate for motility (Sanai *et al*., 2005).

There are also some glioma-related factors that are also associated with NSC. The VEGF, a molecule that stimulates angiogenesis and proliferative potential, is highly produced and secreted by glioma cells (Plate *et al*., 1994). Correspondingly, it was already demonstrated that embryonic NSC secrete diffusible VEGF, which underlies their ability to protect endothelial cells against severe ischemia and promote angiogenesis in ischemic striatum (Roitbak *et al*., 2008). Moreover, S100B, a calcium-binding protein, has also been referred as a biomarker of some pathological conditions, such as brain tumours, since levels of S100B expression are elevated in these disorders (Michetti *et al*., 2012). Therefore, being a chemotactic molecule (Bianchi *et al*., 2011), we can hypothesize that this molecule may also perform a pivotal function in tumour cell invasion and metastasis. Along maturation of astrocytes from NSC, the expression of S100B is increased, suggesting that S100B expression define a late developmental stage after which GFAP-expressing cells lose their NSC potential (Raponi *et al*., 2007). The matrix metalloproteinases (MMP) which have the ability to degrade macromolecules of the ECM, have been described as responsible for tumour invasion and infiltration. In gliomas, the gelatinases MMP, MMP-2 and MMP-9 show elevated levels of expression, facilitating invasion into the surrounding brain and participate in neovascularization (Sun *et al*., 2012). Interestingly, a very recent study has identified MMP-9 as the molecular effector of increased NSC proliferation and migration in lower O_2 concentrations, a condition that more appropriately mimic the environment of the neurogenic stem cell niche in the developing and adult brain (Bianchi *et al*., 2011; Ingraham *et al*., 2011).

Autophagy is the mechanism responsible for degrading long-lived proteins and cytoplasmic organelles, which involves a membranous organelle, the autophagosome (Liu *et al*., 2011). Moreover,

autophagy has a fundamental role in cancer such as in brain tumours. However, perturbations in autophagy can contribute to malignant disease. Hence, autophagy can be both oncogenic and tumour suppressive, indicating that autophagy has different roles at different stages of tumour development. Recent data indicate that this process may play a critical role in the benign to malignant transition, also central to the initiation of metastasis (Macintosh *et al*., 2012). Concerning embryonic development, the roles of autophagy are still largely uncharacterized, but there is some evidence supporting the existence of a complex interplay between autophagy and cell proliferation during mammalian neural development (Fimia *et al*., 2007). Thus, the process of autophagy in NSC or NPC can have a similar role as in glioma cells.

In summary, the following figure (Fig. I.8) present the main features shared by NSC and glioma cells.

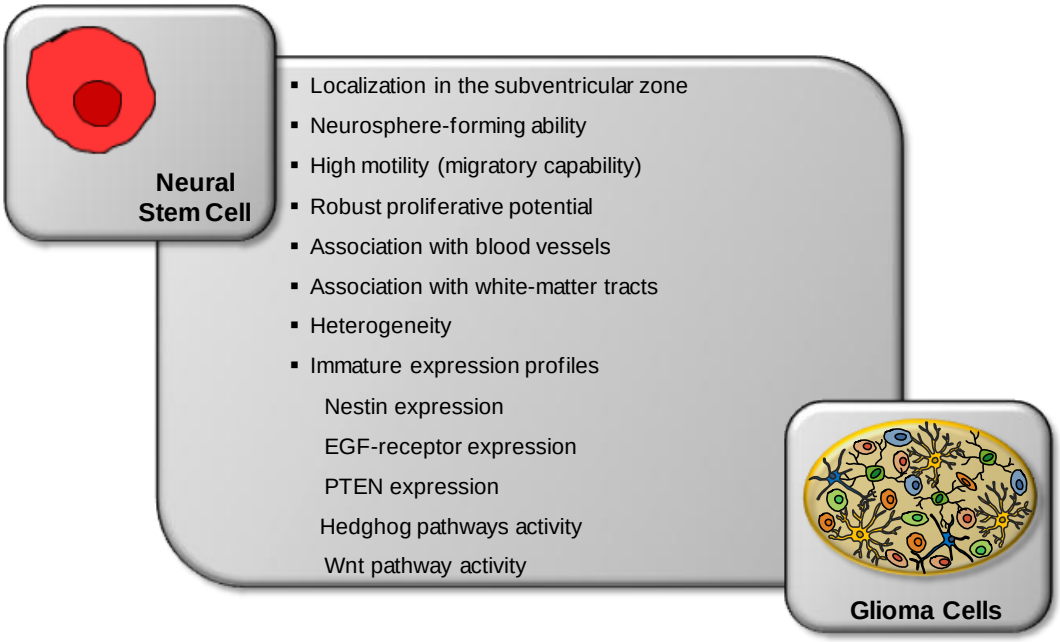


Fig. I.8 – Shared features by neural stem cells and glioma cells. Cancer research is now being motivated to drive its attention to the application of the principles of stem cell biology to the study of human brain tumours, such as gliomas, since neural stem cells (NSC) share common features with a specific population of tumour cells, the cancer stem cells (CSC). This suggests that NSC or early-differentiated cell type lineages are in the origin of gliomas. Addapted from Sanai *et al.* (2005)

It is important to emphasize that although NSPC or early-differentiated cell type lineages are suggested to be in the origin of gliomas, it is not known which developmental stage is more susceptible to the malignant transformation. Thus, it will be interesting to investigate which phenotype, between NSPC and astroglial cells, along the different developmental stages, have the highest tumourigenic potential. By identifying the cell of origin for brain tumours, we will be better equipped to understand how the molecular alterations lead to cancer, and how we can target those alterations by treatment, or prevent them from occurring.

3. Aims

Our general goal was to identify which cell developmental stage, in the NPC differentiation process towards astrocytes, is the phenotype most similar to the glioma one, in order to determine and better comprehend the cellular pathways that might be involved in gliomagenesis.

To accomplish this aim, our initial task is to perform primary neurosphere cultures from mouse brain cortex at E15 and to subsequently induce astroglial differentiation until 7 *days in vitro,* characterizing the cellular population along the differentiation process regarding markers for stem and mature neural cells. Our next task will be to characterize the evolution of some tumour-related factors such as the multidrug resistance, the angiogenesis potential, the autophagy ability and the migratory capability, as well as the release of metalloproteinases and S100B, in (i) NS, (ii) differentiating astrocytes and (iii) in a glioma cell line (GL261). Finally, based on the data obtained, we will identify the developmental stage more similar to the glioma cell line.

In summary, the tasks to be accomplished in the present thesis encompass:

1. Primary neurosphere cultures and subsequent differentiation into astrocytes;
2. Characterization of the different developmental phenotypes;
3. Evaluation of tumour-related factors in glioma cells;
4. Identification of the cell developmental stage phenotype more similar to the glioma cell.

II. MATERIAL AND METHODS

1. Reactives

1.1 Cell cultures media

Dulbecco's modified Eagle's medium (DMEM) was acquired from Biochrom AG (Berlin, Germany). RHB-A^{TM} medium was purchased from Stem Cell Sciences (Cambridge, UK)

1.2 Supplements and chemicals

Heat-inactivated chicken serum, trypsin 2.5% (10×) and 1× Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS) were acquired from Invitrogen (Carlsbad, CA, USA). Antibiotic antimycotic solution (AB/AM) (20×), gelatin 2% (bovine skin), deoxyribonuclease I bovine (DNAse I), Poly-D-Lysine (PDL), Hoechst dye 33258, 2-mercaptoethanol, bovine serum albumin (fraction V, fatty acid free) (BSA), Tris-base, phenylmethylsulfonyl fluoride (PMSF), and propidium iodide [PI; 3,8-diamino-5-(3-(diethylmethylamino) propyl)-6-phenyl phenanthridinium diiodide], Sigma Fast OPD, 5-Bromo-2'-Deoxyiridine (BrdU), Coomassie Brilliant Blue R-250 and trypsin-EDTA solution (10x) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, Germany). Cell lysis buffer® and LumiGLO® were from Cell Signaling (Beverly, MA, USA). Murine EGF and murine bFGF (basic fibroblast growth factor) were obtained from PeproTech (Rocky Hill, NJ, USA). NaOH, HCl, acrylamide, bis-acrylamide, glycine, glucose anhydre, methanol, Giemsa, paraformaldehyde (PFA), and Tween 20 were from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS) was acquired to VWR-Prolabo. Nitrocellulose membrane was obtained from Amersham Biosciences (Piscataway, NJ, USA). Triton X-100 (100 mL) was from Roche Diagnostics (Indianapolis, USA). DPX mountant for microscopy was acquired from BDH Prolabo (Bangkok, Thailand).

1.3 Antibodies

Primary antibodies: Rabbit anti-GFAP (1:500), mouse anti-BrdU (1:750), mouse anti-β-actin (1:5,000) and monoclonal anti-S100B SH-B1 (1:1,000) were acquired from Sigma-Aldrich. Mouse anti-HuC/HuD (1:750) from Molecular Probes (Leiden, The Netherlands). Rabbit anti-NG2 (1:200), mouse anti-MBP (1:200), rabbit anti-Sox2 (1:500), mouse anti-MAP2 (1:100) and mouse anti-nestin (1:200) were obtained from Millipore (Billerica, MA, USA). Rabbit anti-VEGF (1:200 for Western blot assay; 1:100 for immunocytochemistry), mouse anti-VEGFR-2 (or Flk-1, 1:100) and mouse anti-vimentin (1:25) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-LC3B (microtubule-associated protein light chain 3) from Cell Signaling (Beverly, MA, USA). Rabbit anti-GLAST from AbCam (Cambridge, UK) and polyclonal anti-S100B from Dako (Denmark A/S).

Secondary antibodies: Horseradish peroxidise-labelled goat anti-rabbit IgG (1:5,000) was from Santa Cruz Biotechnology and horseradish peroxidise-labelled goat anti-mouse IgG (1:5,000) from

Amersham Biosciences. Alexa Fluor® 594 goat anti-mouse IgG and Alexa Fluor® 488 goat anti-rabbit were acquired from Invitrogen. Fluorescein isothiocyanate (FITC) anti-mouse IgG was obtained from Vector Labs (Burlingame, CA, USA)

2. Equipment

Basic equipment included a fluorescence microscope (model AxioScope A1) with integrated camera (AxioCamHRm), AxioScope HBO50 with integrated camera (Leica, model DFC490) and optical microscope with phase-contrast equipment (Olympus, model CK2-TR), all purchased from Carl Zeiss, Inc. (North America). For Western blot and Zymography assays it was used the Mini-PROTEAN 3 Multi-Casting Chamber (Bio-Rad Laboratories). For protein quantification it was used a Microplates reader (PR 2100 Microplate Reader, Bio-Rad Laboratories). For metalloproteinases gels photos it was used the Chemidoc (Bio-Rad Laboratories). Samples sonication for a well homogenization was performed in the Ultrasonic Processor UP100H (Hielscher-Ultrasound Technology, Teltow, Germany). To ensure a stable ambient to optimal cell growth (37°C and 5% CO_2), cell cultures were maintained in HERAcell 150 incubator (Thermo Scientific, Waltham, MA, USA) and execution was accomplished in sterile conditions using a Holten Lamin Air HVR 2460 (Allerod, Denmark). For flow cytometry studies, we used the Guava – Easy Cyte HT model (Millipore). A 48-well microchemotaxis chamber (Boyden chamber) and polycarbonate track-etch membranes with polyvinylpyrrolidone (PVP) treatment were purchased from Neuro Probe, Inc (Gaithersburg, MD, USA).

3. Methods

3.1 Cell cultures and treatments

3.1.1 Primary neurosphere culture of mouse brain cortex at E15

Animal care followed the European Legislation on Protection of Animals Used for Experimental and Scientific Purposes (EU directive L0065, 22/07/2003) in order to ensure their well-being and minimize animal´s use and suffering.

Cortical neural precursors were isolated from E15. Briefly, pregnant female mice (CD1, Harlan Laboratories, Inc., Indianapolis, USA) at gestational stage E15 were euthanized by asphyxiation with CO_2. The fetuses were rapidly decapitated and after removal of meninges and white matter, the neocortices were collected in 9 mL of HBSS and mechanically fragmented. After chemical dissociation with trypsin-EDTA 10% and DNAse I (1 U/mL), the suspension was incubated for 30 min at 37ºC, with occasional mixing. Following trypsinization, cells were washed three times with HBSS and resuspended in RHB-A™ medium. Cells were then mechanically dissociated using a Pasteur pipette

performing more or less 20 passages. Around $1x10^6$ cells/mL were plated onto 24-well uncoated tissue culture plates in RHB-A™ medium supplemented with 10 ng/mL of recombinant murine EGF and bFGF, to form free-floating NS, maintained at 37ºC in a humidified atmosphere of 5% CO_2. After 48 h, the NS were removed, centrifuged and resuspended in fresh RHB-A™ medium with EGF and bFGF. This cellular suspension was plated onto 96-well uncoated tissue culture plates for more 48 h under the same temperature and moisture conditions. After this incubation period, a first set of NS was removed and chemically dissociated by addition of trypsin 0.1% and mechanically dissociated through passages in a pipette. Approximately $7,0x10^5$ cells/mL were incubated in same culture conditions (RHB-A, EGF, bFGF) and plated onto culture plates (some of them containing glass coverslips coated with PDL) at 37ºC, in a humidified atmosphere of 5% CO_2, during appoximately four hours. After that, cells plated in coverslips, were fixed with freshly prepared 4% PFA during 20 min and used for immunocytochemistry assays, and cells in the wells without coverslips were used for flow cytometry studies or lysed for Western blot, while growth medium was removed, centrifuged and stored at -80°C to evaluate S100B and MMP contents.

A second set of NS were maintained till astroglial differentiation as described in the next Section.

A schematic overview of cortical neural precursor's proliferation as floating aggregates and cell treatments is depicted Fig. II.1A.

3.1.2 Neurospheres *in vitro* astroglial differentiation

NS were rinsed with PBS and dissociated with 1% trypsin by forcing several passages through a pipette. After dissociation, single cells were induced to differentiate into astrocytes by plating them onto culture plates (some of them containing glass coverslips) in a concentration of approximately $7,0x10^5$ cells/mL, in DMEM medium supplemented with 10% FBS. Cells were maintained in differentiating conditions during 7 days *in vitro* (DIV), without changing the culture medium. Each day of the astroglial differentiation process, cells plated in coverslips were fixed with freshly prepared 4% PFA during 20 min and used for immunocytochemistry assays. Collection of the cells was performed at several time intervals to be evaluated and compared to those of the glioma phenotype. We decided to use 1, 2, 3 and 7 DIV. Cells were then used for flow cytometry studies or lysed for western blot and their growth medium was removed, centrifuged and stored at -80°C to analyze the respective supernatant content in S100B and MMP.

A schematic overview of astroglial differentiation from NS is presented in Fig. II.1B.

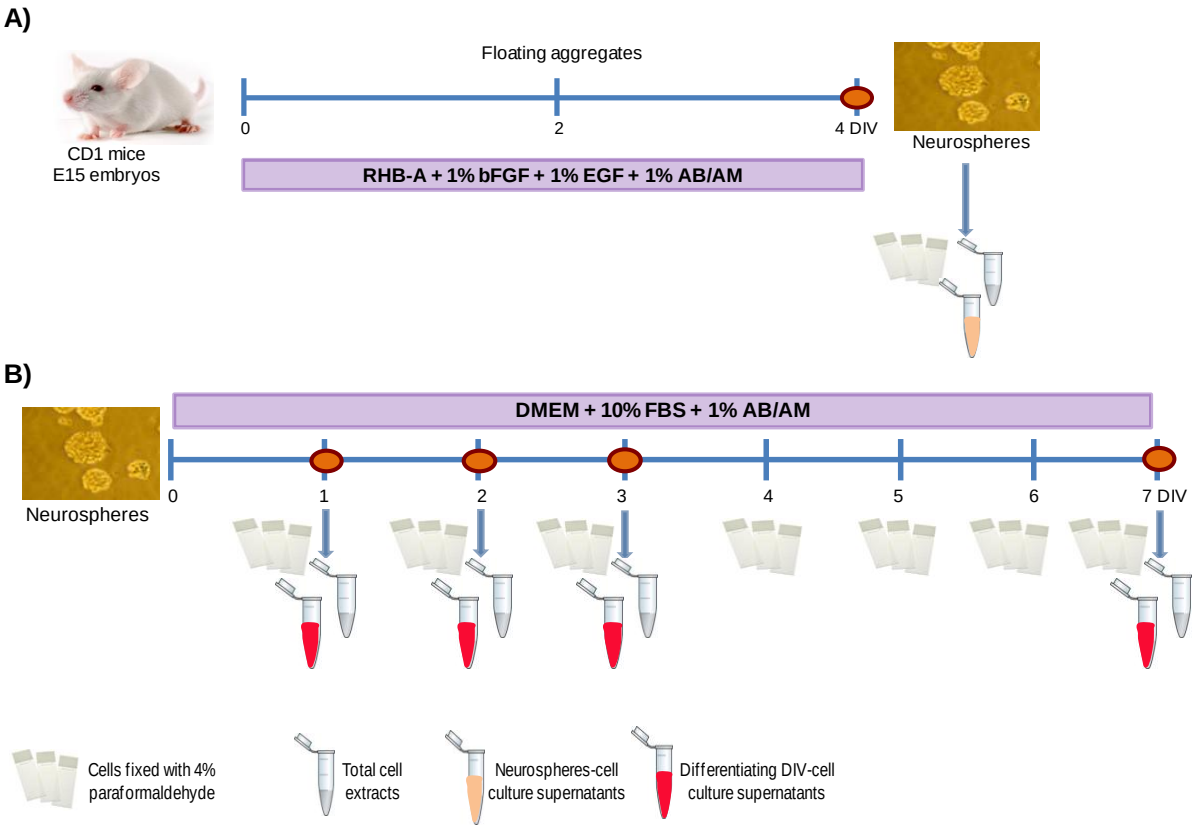


Fig. II.1 - Schematic representation of the experimental model, involving the preparation of primary neurosphere culture and the induction of astroglial differentiation. A) Neural precursor cells were obtained from embryonic day 15 (E15) CD1 mouse brain cortex, growing as neurospheres in RHB-A medium, in the presence of the growth factors epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (10 ng/mL). After 4 days *in vitro* (DIV), cells were fixed or lysed for western blot analysis and the respective cell-free medium was collected. B) Astroglial differentiation was induced by 10% fetal bovine serum (FBS) and the cells were cultured until 7 DIV. At 1, 2, 3 and 7 DIV, cells were fixed or lysed for western blot analysis and the respective cell-free medium was collected. Cells fixed in 4% paraformaldehyde were used for immunocitochemistry assay toward the evaluation of several cellular markers, proliferative potential, vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor 2 (VEGFR-2) (the last two only on NS, 1, 2, 3 and 7 DIV). Total cell extracts from NS, 1, 2, 3 and 7 DIV in differentiating conditions were used for Western blot assay to evaluate the multidrug resistence-associated protein (Mrp1), VEGF and microtubule-associated protein light chain 3 (LC3). The cell culture supernatants from NS, 1, 2, 3 and 7 DIV in differentiating conditions (salmon from NS, and red from differentiating DIV) was used for S100B and matrix metalloproteinases (MMP) evaluation. AB/AM, Antibiotic antimycotic solution; DMEM, Dulbecco's modified Eagle's medium

3.1.3 GL261 mouse glioma cell line

The GL261 was a kind gift from Dr Geza Safrany, from the National Research Institute for Radiobiology and Radiohygiene, in Hungary. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 38.9 mM glucose, 11 mM sodium bicarbonate, 1% penicillin/streptomycin and 10% FBS, at 37ºC and maintained in 5% CO_2 conditioned atmosphere during 5 DIV. After this period, cells were lysed for Western blot and their growth medium was removed, centrifuged and stored at -80^{o}C to analyze the respective supernatant S100B and MMP contents.

The cell culture described above was performed by another master student from our lab.

3.2 Immunocytochemistry

For characterization of the NS and the differentiating cells, an immunofluorescent detection of immature/progenitor cells, astrocytes, neurons and oligodendrocytes was performed. Fixed cells were incubated with a glycine solution during 10 min at room temperature (RT) and permeabilized with 0.1% triton solution for 10 min. Cells were then incubated with a blocking solution [10% FBS in Tween 20-Tris buffered saline (T-TBS, 0.05% Tween 20, 20mM Tris-HCl, 500 mM NaCl, pH 7.5)] for 30 minutes at RT. Cells were incubated overnight, at 4°C, with anti-Sox2 antibody (rabbit, 1:500) and anti-nestin antibody (rabbit, 1:200) to stain the presence of undifferentiated/immature cells. To identify astrocytes, it was used anti-vimentin (mouse, 1:25) antibody (progenitor glial cells) as well as anti-GFAP (rabbit, 1:500) and anti-GLAST (rabbit, 1:500) antibodies (differentiated astrocytes). To evaluate the presence of oligodendrocytes, it was used an anti- neural/glial antigen 2 (NG2) (rabbit, 1:200) and anti-myelin basic protein (MBP) (mouse, 1:200) antibodies, to evaluate the presence of progenitor and mature cells, respectively. To stain progenitor and mature neurons we used an anti-HuC/D (mouse, 1:750) and anti-microtubule-associated protein 2 (MAP-2) (mouse, 1:100) antibodies, respectively. For the determination of VEGF and VEGFR-2 (tumour-related factors), it was used an anti-VEGF (rabbit, 1:100) antibody and an anti-VEGFR-2 (mouse, 1:100) antibody, respectively. Cells were then incubated with a species-specific fluorescent secondary antibody labeled with FITC (green fluorescence), Alexa Fluor® 594 goat anti-mouse IgG, or Alexa Fluor® 488 goat anti-rabbit, for 90 min at RT. For nuclei staining, coverslips were stained with Hoechst dye 33258 during 2 min (Falcao *et al.*, 2005). Following a final rinse in T-TBS [0.2% Tween 20, 20 mM Tris-HCl (pH 7.5), 500 mM NaCl] and dehydration with methanol, coverslips were mounted onto uncoated glass blades using DPX mountand and stored at 4°C. Finally, pairs of U.V. and fluorescence images of ten random microscopic fields (original magnification: 252x) were acquired per sample (Falcao *et al.*, 2007). Immune-positive cells for each cell type and total cells were counted to determine the percentage of positive nuclei. The resultant values were presented as percentage of positive cells for each staining.

Table II.1 summarizes the most frequent markers used in the identification of the several cell types along differentiation.

Table II.1 – Proteins markers of interest for the characterization of each differentiating neural cell type.

Cellular type		Protein
Neural stem/progenitors	-	Sox2 Nestin
Astrocytes	Precursor	Vimentin
	Differentiated	GFAP GLAST
Oligodendrocytes	Precursor	NG2
	Differentiated	MBP
Neurons	Precursor	HuC/D
	Differentiated	MAP2

3.3 Proliferative potential

The proliferative potential of NS and of differentiating cells was determined by quantification of BrdU-positive cells. BrdU is a synthetic thymidine analog that incorporates into the DNA when the cell is dividing (during the S-phase of the cell cycle). Some of the cells that were plated in coverslips were previously incubated with 10 µM BrdU during 3 h, and fixed with 4% of PFA, followed by 30 min treatment with HCl 1N at 37ºC. For detection of $BrdU^+$ cells identification, we performed thereafter an incubation with an antibody against BrdU (mouse, 1:750), and subsequently with a secondary antibody (anti-mouse, FITC).

Another staining methodology was used in parallel to afford a more precise quantification. For that, NS and the differentiating cells were removed by tripsin action, counted and distributed to five different eppendorfs with an approximate cellular concentration of $5{,}0x10^5$ cells. After their centrifugation at 500 *g* (4^oC), cells were fixed with 4% of PFA in PBS during 20 min on ice. After another similar centrifugation, supernatants were removed and cells rinsed with PBS. Fixed cells were again centrifuged, and after removal of supernatants cells were incubated for 30 min with HCl 1N at 37ºC for 30 min. After centrifugation, cells were incubated for 20 min with blocking solution (10% FBS in T-TBS) on ice. Cells were again centrifuged and incubated with anti-BrdU antibody (mouse, 1:750) for 30 min at 4ºC. After new centrifugation, cells were incubated for 20 min, on ice, with an anti-mouse antibody labeled with FITC (1:227). After centrifugation, cells were rinsed once and ressuspended in PBS. Finally, cellular suspension was plated in a 96-wells plate and submitted to flow cytometry by using GUAVA. Results were expressed as percentage of $BrdU^+$ cells.

3.4 Western blot assay

Brieffly, total cell extracts were obtained by lysing cells in ice-cold 1x Cell Lysis Buffer plus 1 mM PMSF for 10 min, on ice and with shaking, followed by sonication during 20 seconds. The lysate was centrifuged at 14,000*g* for 10 min, at 4^oC, and the supernatants were collected and stored at -80^oC. Protein concentration was determined using the Bradford method (Bradford, 1976). Equal amounts of protein content were subject to 12% (for LC3B evaluation) or 8% (for Mrp1 and VEGF determination) sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE), running with fixed voltage of 80 *V* for 12% gel and fixed amperage of 50 mA for 8% gel. After running the gel, proteins were transferred to a nitrocellulose membrane, and blotted membranes were rinsed once with T-TBS and blocked for 1 h at RT with 4% milk in T-TBS for Mrp1 detection and 5% milk T-TBS for LC3B and VEGF evaluations. Membranes were then incubated overnight at 4ºC with a specific Mrp1 rabbit antibody (1:750 in blocking solution), a specific LC3B rabbit antibody (1:1000 in 5% of BSA in T-TBS solution) and a specific VEGF rabbit antibody (1:200 in 5% BSA in T-TBS solution). After washing three times with T-TBS, the membranes were incubated with HorseRadish Peroxidase (HRP)-labeled anti-rabbit (1:5,000) in blocking buffer, for 1 h, at RT. Again, after washing membranes with T-TBS, chemiluminescent detection was performed by LumiGLO® and bands were visualized by autoradiography with Hyperfilm ECL. The relative intensities of protein bands were analyzed using the

Image Lab™ analysis software, after scanning with Chemidoc, both from Bio-Rad Laboratories (Hercules, CA, USA) (Fernandes *et al*., 2006).

3.5 Gelatin Zymography

The assay was performed as previously described by Silva *et al*. (2010). MMP quantification was performed through the gelatin zymography method, in which the protease activity is directly observed in the running gel based on the absence of color (white bands), at the particular site of protease action. For this determination, aliquots of culture supernatants were analyzed by SDS-PAGE zymography in 0.1% gelatin/10% acrylamide gels under non-reducing conditions, at 20 mA/gel. After electrophoresis, gels were washed for 1 h with 2.5% Triton X-100 (in 5 mM Tris pH7.4; 5 mM $CaCl_2$; 1 µM $ZnCl_2$) to remove SDS and renature the MMP species in the gel. Then, gels were incubated overnight in the developing buffer (5 mM Tris pH7.4; 5 mM $CaCl_2$; 1 µM $ZnCl_2$) to induce gelatin lysis. For enzyme activity analysis, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 and distained in 30% ethanol/10% acetic acid/H_2O. Gelatinase activity, detected as a white band on a blue background, was photographed in Chemidoc and analyzed using the Image Lab™ software. Results were normalized with total protein concentration (Silva *et al*., 2010).

3.6 ELISA

S100B concentration released into the culture media was determined by ELISA (enzyme-linked immunosorbent assay). Initially, 100 µl of a capture antibody (monoclonal antibody anti-S100B SH-B1, diluted at 1:1000 in carbonate-bicarbonate buffer 50 mM, pH 9.5) was placed in each well of a 96-well plate and incubated overnight at 4°C. After this incubation period, the 96-wells plate was rinsed three times with wash buffer (0.1% BSA with 0.05% Tween) and incubated with blocking solution (2% BSA in PBS) for 1 h at RT. The plate was again washed once with the buffer and samples added and incubated for 2 h at 37ºC. Following three washes with wash buffer/well, samples were incubated with the detection antibody (polyclonal antibody anti-S100B, diluted at 1:5,000 into 0.5% BSA with 0.2 mM $CaCl_2$ in PBS) for 30 min at 37°C. Finally, after three new washes, an anti-rabbit peroxidase-conjugated antibody (1:5,000) was added for further 30 min at 37ºC. Then, more three washes were done with wash buffer. The 96-well plate was washed once more with PBS, and the substrate solution was added and followed by an incubation of 30 min at RT (Leite *et al*., 2008). The colorimetric reaction with Sigma Fast OPD tablets was measured at 492 nm, using the microplate reader and results were expressed in ng/mL.

3.7 Migration Assay

This method was originally adapted from the protocol reported by Nolte *et al*. (1996) and, after several attempts and failures, some modifications led to the optimization of this method by our group.

The migratory potential was evaluated only in NS and differentiating cells with 1 and 7 DIV and it was compared with the one of glioma cells. NS in suspension were centrifuged, dissociated with the aid of tripsin for 1 min at 37°C, counted and plated accordingly to the schematic representation of Fig.II.2. The remaining cells were washed once with PBS and incubated with trypsin for 2 min at 37°C, centrifuged, counted and plated as depicted in Fig. II.2.

Cell migration assays were performed in a 48-well microchemotaxis chamber. Initially, each of the bottom wells of the Boyden chamber was loaded with 25 µl of VEGF solution (20 ng/mL in RHB-A or DMEM medium) or 25 µl of glioma growth media (see Fig.II.2). Thereafter, a PVP-treated polycarbonate filter (8 µm pore size), previously moistened with RHB-A or DMEM, was placed over bottom wells. After addition of the upper plate, both bottom and upper wells were only separated by the membrane between them. The cells ($1.5x10^4$ cells/well) were thoroughly added in 50 µl of RHB-A (for neurospheres) or DMEM (for differentiating astrocytes and glioma cells) and incubated at 37°C for 5 h (Schmidt *et al*., 1999). The filter was removed, fixed with methanol during 3 min and after drying, stained for 30 min at RT with freshly prepared and filtered 10% Giemsa in phosphate buffer. Finally, the excess color from the side filter without cells (upper) was carefully clean and the filter/membrane was mounted on a glass slide. Image of one microscopic field (original magnification: 100X) was acquired per well using a Leica DFC490 camera adapted to an AxioSkope® microscope and the number of cells was counted. On each experiment at least three wells were sampled per condition.

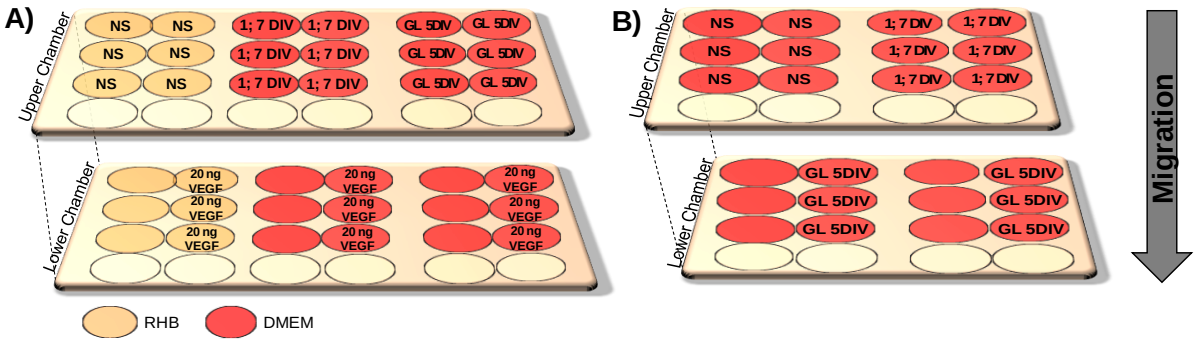


Fig. II.2 – Schematic representation of the migration assay. A) For the determination of migration levels to vascular endothelial growth factor (VEGF), neurospheres (NS) in RHB-A™ medium (orange), 1 and 7 days *in vitro* (DIV) differentiating astrocytes in DMEM (Dulbecco's modified Eagle's medium) media (red) as well as glioma (GL) cells with 5 DIV in DMEM media (red) were plated in the upper chamber, while in the lower chamber (in corresponding wells) it was only added media (control) or 20 ng of VEGF in media. B) For the determination of migration cell capacity to conditioned glioma cells media, NS, 1 and 7 DIV differentiating astrocytes were ressuspended in DMEM and plated in the upper chamber, while in the lower chamber (in corresponding wells) it was only added DMEM (control) or media from glioma cells with 5 DIV. All experiments were performed in triplicate.

4. Statistical analysis

Results of at least, three different experiments performed in duplicate, were expressed as mean ± SEM. Differences between groups were determined by one-way ANOVA with Dunnett's multiple comparisons post tests, using Instate 5.01 GraphPad software (San Diego, USA). It was considered $p < 0.05$ as statistically significant.

III. RESULTS

NSC have been described as a potential origin of some brain tumours. Due to the sharing of several common features between NSC or NPC and glioma cells [such as the longevity, high motility, proliferative capacity (Ignatova *et al*., 2002; Sanai *et al*., 2005), and pluripotency (Louis *et al*., 2007; Tan *et al*., 2006)] it is thought that gliomas (particularly astrocytomas) can be originated by malignant transformation of NSC or NPC, or by the occurrence of modifications in the normal course of differentiation of glial NPC (Sanai *et al*., 2005). This malignity may be related with a phenotype that will most resemble glioma cells. Thus, it was evaluated some tumour-related factors to investigate which developmental stage, in the NSC differentiation process towards astrocytes, possessed the highest number of tumourigenic characteristics most similar to glioma cells.

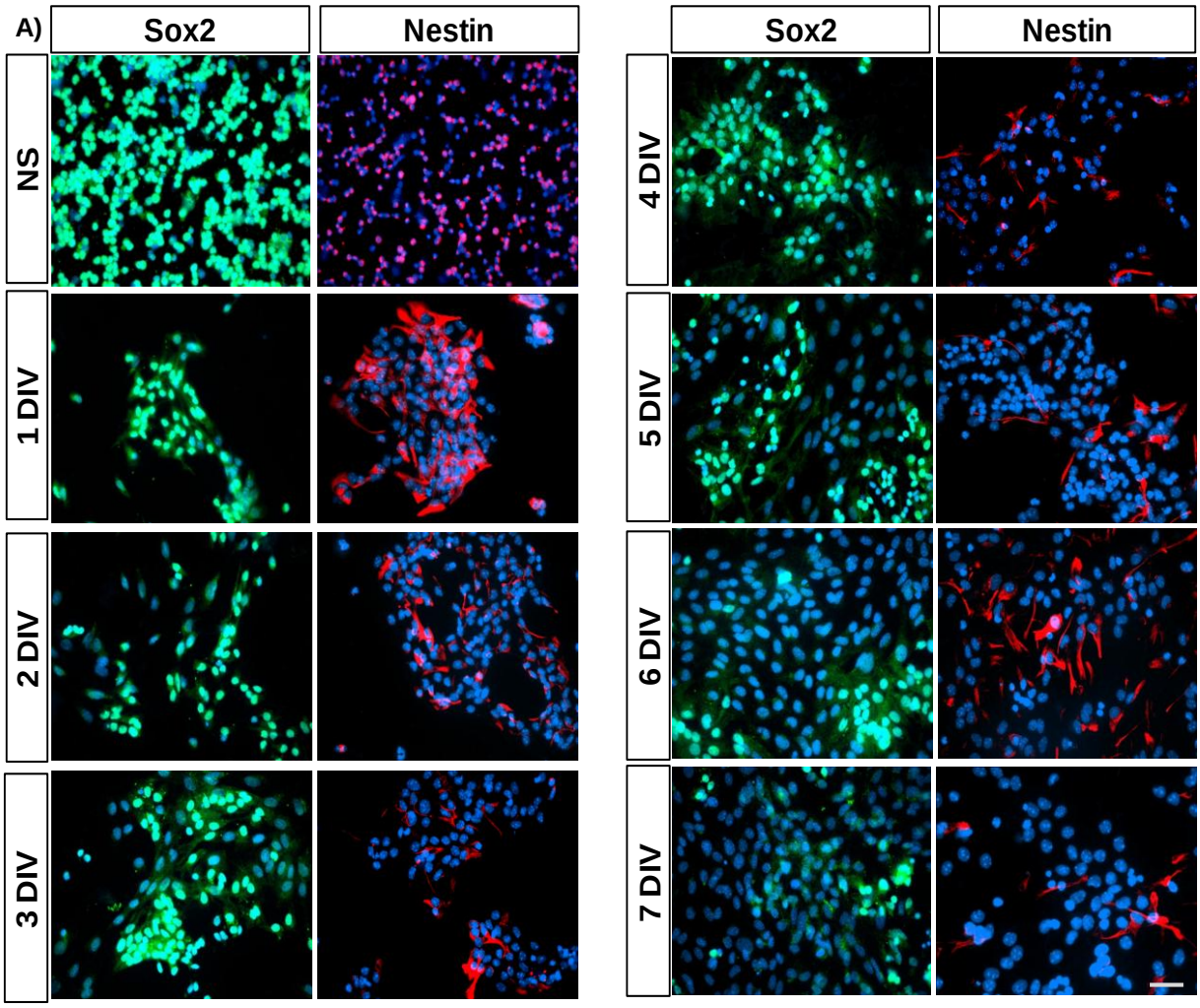
1. Characterization of neurospheres and of the different developmental phenotypes

Primary cultures of NS obtained from CD1 mice cortices at E15 were cultured in the proliferation media and induced to differentiate into astrocytes, as described in methods. NS were characterized regarding their cellular composition and concerning the phenotype originated throughout the differentiation process into astrocytes, by immunocytochemistry assays. The results reflected the efficacy of the differentiation method and allowed us to identify and select the phenotypes to be used in the tasks that will follow.

1.1 Differentiation of NS into astrocytes leads to a reduction in the undifferentiated cell markers Sox2 and nestin

The percentage of an immature phenotype in NS and differentiating cells was determined using antibodies against undifferentiated/immature cell markers, such as Sox2 and nestin. We could observe that the expression of both proteins decreased throughout differentiation (Fig. III.1). The expression of these two proteins was more abundant in the NS and gradually was reduced with the advance of differentiation. Regarding specifically Sox2, there was a regular decline through the differentiation process, culminating in a 50% decrease at 7 DIV cells ($p<0.01$). For nestin, there was also an overall reduction; however, this reduction was very abrupt from 1 DIV to 2 DIV ($p<0.01$) followed by a continuously decrease. As expected, while the Sox2 fluorescent staining (green) localized in the nuclei, the majority of nestin (red) protein was spread in the cytoplasm.

Overall, these results indicate that the number of immature cells decrease along differentiation, and that nestin expression is more rapidly reduced when compared with the Sox2 one.



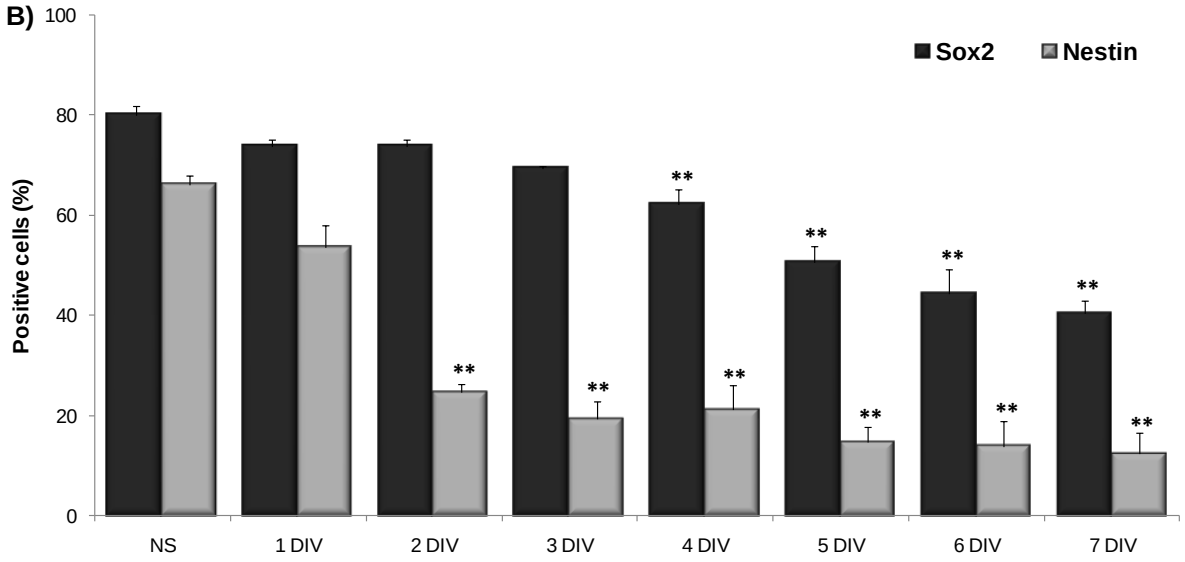


Fig. III.1 – Expression of immature phenotypes during proliferation and along differentiation. Cells were cultured as indicated in methods. A) Nuclei were stained with Hoechst dye (blue) and cells labeled for Sox2 (green) and Nestin (red). Representative results from one experiment are shown. B) Graph bars represent the percentage of cells (mean ± SEM) positive for Sox2 (black) and nestin (grey), relatively to the total number of nuclei. Data were obtained from at least three independent experiments. **p<0.01 *vs.* neurospheres (NS). DIV, days *in vitro* under differentiating conditions. Scale bar represents 60 µm.

1.2 Differentiation of NS into astrocytes leads to a reduction of BrdU, a marker of proliferating cells

The proliferative potential was evaluated through the incubation of cells with BrdU and further immunostaining with an antibody against BrdU, in order to determine the percentage of the BrdU-positive cells in NS and along astrocyte differentiation. The image presented in Figure III.2.A shows that the number of BrdU-positive cells decreases along differentiation. Based on the results obtained in the previous section, the immature cells are those showing the highest proliferative potential. There is an evident decrease in proliferation from NS stage to 1 DIV cells (0.5-fold, $p<0.01$), followed by a more gradual and less pronounced reduction in BrdU-positive cells from 1DIV to 7 DIV. Immunostaining results (Fig. III.2B) were further corroborated when the flow cytometry (GUAVA) method was used (Fig.III.2C), which showed a similar result profile.

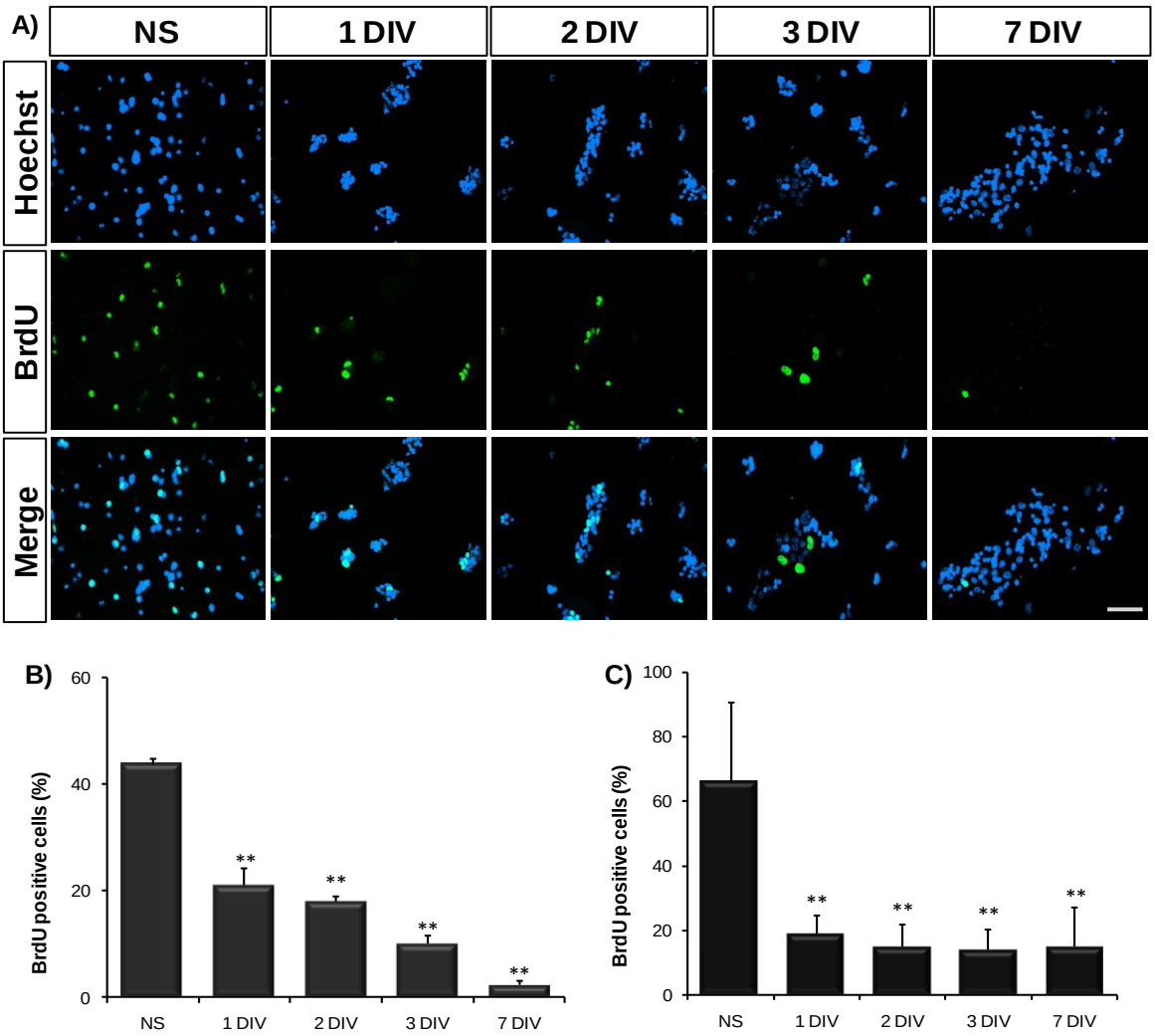


Fig. III.2 – Expression of proliferative potential during cell division and differentiation. Cells were cultured and treated as indicated in methods. A) Results obtained from immunocytochemistry assay. Nuclei were stained with Hoechst dye (blue) and cells labeled for 5-bromo-2'-deoxyuridine (BrdU, green). Representative results from one experiment are shown. B) Graph bars represent the percentage of cells (mean ± SEM) positive for BrdU, relatively to the total number of nuclei. Data obtained from at least three independent experiments. C) Results obtained from flow cytometry (GUAVA) evaluation. Graph bars represent the percentage of cells (mean ± SEM) positive for BrdU. Data obtained from at least three independent experiments. **p<0.01 *vs.* neurospheres (NS). DIV, days *in vitro* under differentiating conditions. Scale bar represents 60 µm.

1.3 Differentiation of NS into astrocytes leads to a reduction in the early astrocytic progenitor marker vimentin and an increase in the astrocytic markers GFAP and GLAST

The number of cells with astrocytic features cells in NS and differentiating stages was determined using antibodies against vimentin, to identify astrocytic-progenitor cells, as well as against GFAP and GLAST to identify astrocytes. It was observed that the expression of vimentin decreased throughout differentiation, while the expression of GFAP and GLAST increased. Immunocytochemistry showed that the localization of all proteins was cytoplasmic (Fig. III.3). Regarding the number of vimentin-positive cells, although no significant differences, it gradually increased until 2 DIV (1.3-fold *vs.* NS), decreasing almost 50% thereafter, a value that was maintained from 5 to 7 DIV. Interestingly, although GLAST-positive cells increased more rapidly than the number of GFAP-positive cells (maximal levels at 7 DIV), the incremental peak achieved at 7 DIV was only 2.8-fold higher ($p<0.01$) than the one obtained in NS, while that for GFAP-positive cells was 3.8-fold higher ($p<0.01$). These results demonstrate that the cell population at 7 DIV are mostly cells with astrocytic features and that the astrocytes progenitors are replaced by mature-astrocytes throughout differentiation, since the number of differentiated astrocytes increases appreciably when the number of vimentin-positive cells decrease. Overall, these results reveal that our differentiation method into astrocytes was efficient and appropriate to the intended objective of obtaining this preferential cell type.

1.4 Differentiation of NS into astrocytes leads to an highly decrease in the oligodendroglial markers NG2 and MBP

The number of cells with oligodendroglial features in both NS and differentiating cells was determined using the antibodies against oligodendroglial progenitor cells and oligodendroglial mature cells markers, NG2 and MBP (myelin basic protein), respectively. It was observed that the expression of both proteins undergoes a prominent decrease throughout differentiation and that the localization of the both proteins was cytoplasmic (Fig. III.4). The number of oligodendroglial progenitors (NG2-positive cells) was higher in NS (approximately 13% of positive cells) than the percentage registered along differentiation ($p<0.01$). On the other hand, MBP-positive cells (mature oligodendroglia) appear only at 3 DIV with a percentage less than 1%. This low percentage is observed throughout all the differentiation process in spite of a slight increase from 3 to 5 DIV. This evaluation allowed us to verify that oligodendrocyte-like cells are scarcely represented along the differentiation process.

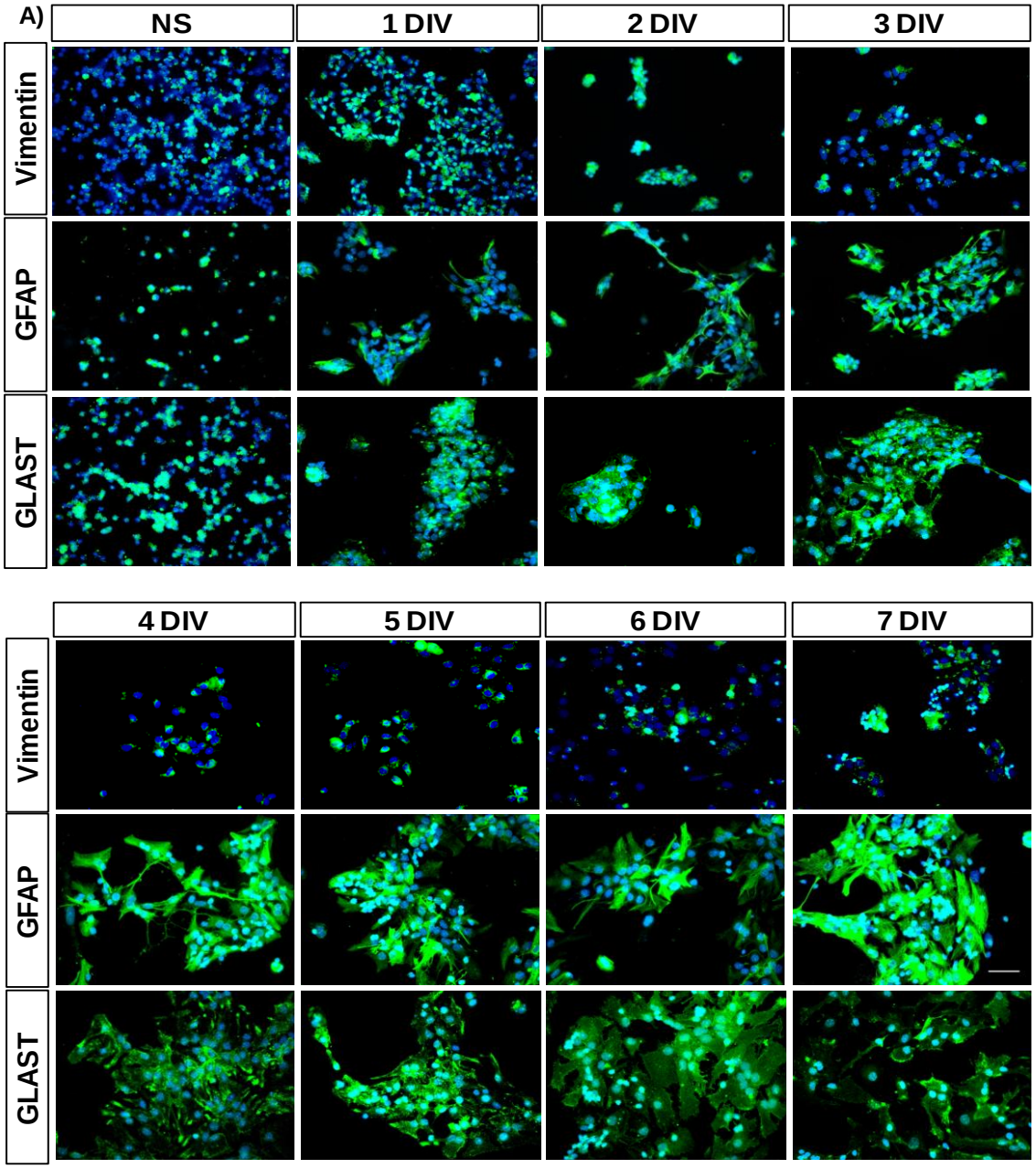


Fig. III.3 – Vimentin-expressing astrocytes-progenitor and GFAP and GLAST-expressing astrocytes observed during proliferation and along differentiation. Cells were cultured as indicated in methods. A) Nuclei were stained with Hoechst dye (blue) and cells labeled for vimentin, glial fibrillary acid protein (GFAP) and glutamate aspartate transporter (GLAST). Representative results from one experiment are shown. B) Graph bars represent the percentage of cells (mean ± SEM) positive for vimentin, GFAP and GLAST, relatively to the total number of nuclei. Data obtained from at least three independent experiments. *p<0.05 and **p<0.01 *vs*. neurospheres (NS). DIV, days *in vitro* under differentiating conditions. Scale bar represents 60 µm.

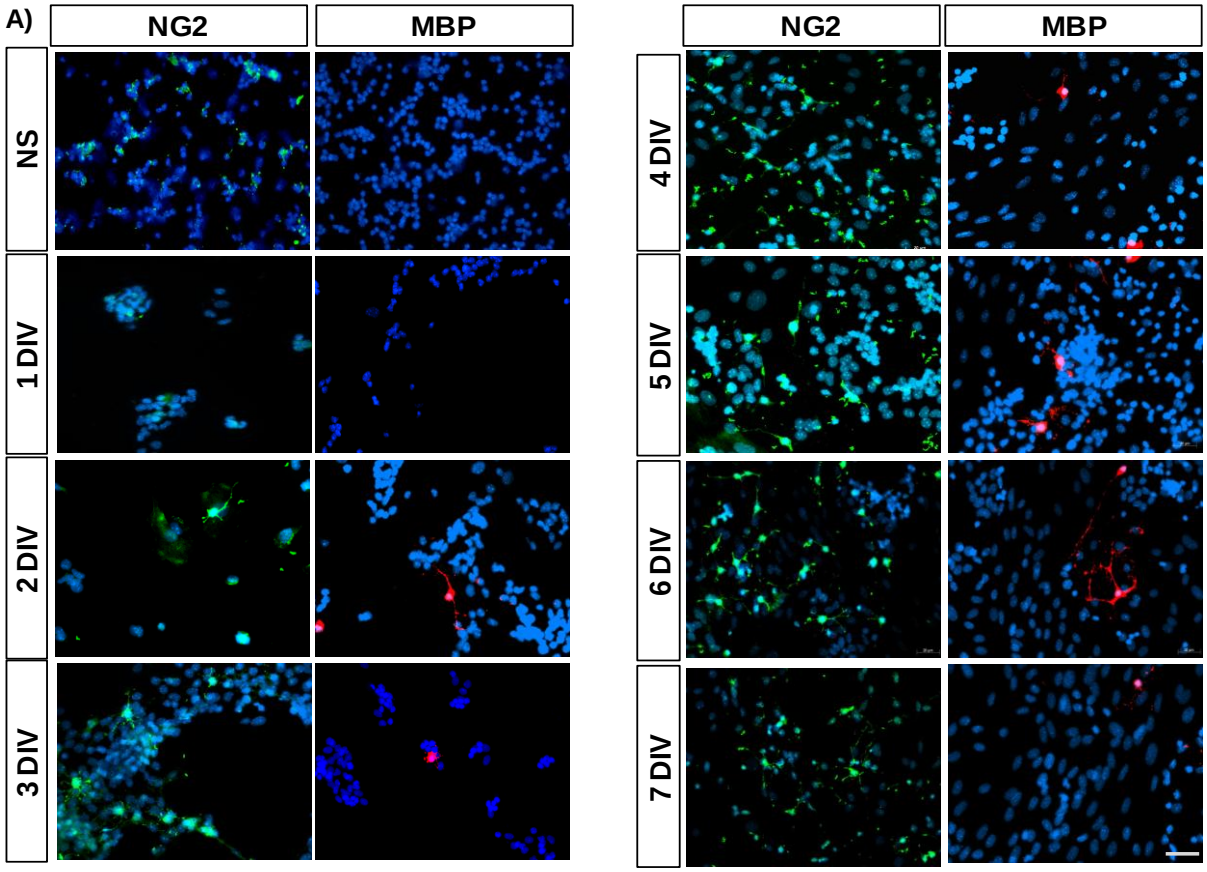


Fig. III.4 – Expression of the oligodendroglial phenotypes during proliferation and along differentiation. Cells were cultured as indicated in methods. A) Nuclei were stained with Hoechst dye (blue) and cells labeled for NG2 (oligodendroglial progenitors, green) and myelin basic protein (MBP, mature oligodendrocytes, red). Representative results from one experiment are shown. B) Graph bars represent the percentage of cells (mean ± SEM) positive for NG2 and MBP, relatively to the total number of nuclei. Data obtained from at least three independent experiments. *p<0.05 and **p<0.01 *vs.* neurospheres (NS). DIV, days *in vitro* under differentiating conditions. Scale bar represents 60 µm.

1.5 Differentiation of NS into astrocytes leads to a highly decrease in the neuronal markers HuC/D and MAP2

The percentage of cells with neuronal features in both NS and differentiating stages were determined using antibodies against neuronal progenitor cells (HuC/D) and against neuronal mature cell markers (MAP2), respectively. Immunocytochemistry showed that the cellular localization of the both proteins were different, i.e., while HuC/D (red) had a nuclear localization, the MAP2 (green) was noticed at the cytoplasm and stained the neuronal ramifications (Fig. III.5). As expected, both the number of neuronal progenitor cells (HuC/D-positive cells), as well as the number of MAP2 positive

cells was low in NS (approximately 5% of positive cells). In addition, both proteins revealed a similar expression profile, but at different time points along differentiation. Thus, for HuC/D, it was observed an increase in the number of positive cells until 4 DIV (7.0-fold *vs.* NS), followed by a reduction that achieved the lower value at 7 DIV (2.0 fold *vs.* NS). On the other hand, the number of MAP2-positive cells that slightly increased up to 2.0-fold, as compared to NS at 1 DIV (ns) was also followed by a decrease till 6 to 7 DIV. These results evidence that despite the presence of neuronal progenitors along all the differentiation process (particularly at 4 DIV), it seems that these cells are not able to further differentiate into mature neurons, as the number of MAP2 positive cells was maintained always very low.

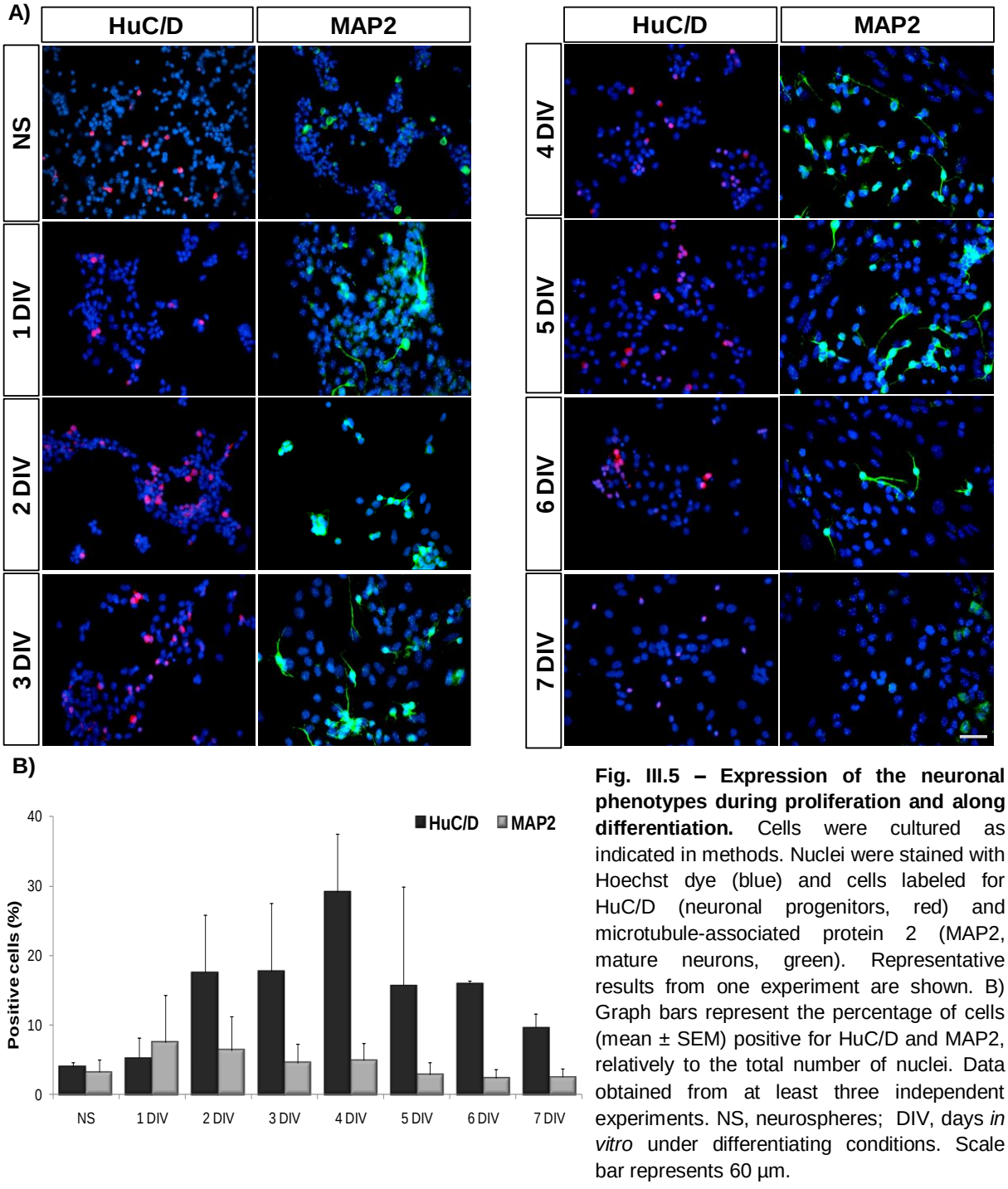


Fig. III.5 – Expression of the neuronal phenotypes during proliferation and along differentiation. Cells were cultured as indicated in methods. Nuclei were stained with Hoechst dye (blue) and cells labeled for HuC/D (neuronal progenitors, red) and microtubule-associated protein 2 (MAP2, mature neurons, green). Representative results from one experiment are shown. B) Graph bars represent the percentage of cells (mean ± SEM) positive for HuC/D and MAP2, relatively to the total number of nuclei. Data obtained from at least three independent experiments. NS, neurospheres; DIV, days *in vitro* under differentiating conditions. Scale bar represents 60 µm.

Collectively, the results obtained for cellular characterization allowed the selection of the stages NS, 1, 2, 3 and 7 DIV as the best to be used in the experiments to be realized in the next task. We excluded the time points 4, 5 and 6 DIV because no significant changes were obtained from 3 DIV and 7 DIV regarding the markers that we used. Moreover, as shown, the values found for 4, 5 and 6 DIV were very similar, thus turning irrelevant further evaluations on these phenotypes, and also considering that the largest modification for astrocytes markers was obtained from 3 DIV to 7 DIV, thus attesting the high prevalence of mature astrocytes.

2. Comparison between tumour-related factors in glioma cells and in the successive developmental phenotypes from neurospheres

For this part of the work it was used not only the cells obtained from primary NS, and cells from the subsequent steps of differentiation (described in the previous section), but the mouse glioma cell line GL261, as well. This cell line is representative of a carcinogen-induced mouse syngeneic glioma model (Newcomb and Zagzag, 2009). The GL261 tumour was originally induced by intracranial injection of 3-methylcholantrene into C57BL/6 mice and maintained by serial intracranial and subcutaneous transplantations of small tumour pieces on the syngeneic mouse strain (Ausman *et al*., 1970; Szatmari *et al*., 2006). These cells have invasive and angiogenic properties and present growth features similar to the human GBM, thus representing an important tool to study the biology of this type of human cancer (Newcomb and Zagzag, 2009).

2.1 Cells from NS reveal levels of Mrp1 expression very similar to those of glioma cells

The Mrp1 expression levels in NS, differentiating cells and GL261 was determined by Western blot assay, using an antibody against Mrp1, normalized against the corresponding β-actin levels. It was observed that the expression of Mrp1 was significantly higher in NS as compared to differentiating cells ($p<0.01$, except in 7 DIV) (Fig. III.6). Thus, the expression profile revealed a decrease of Mrp1 from NS to 1 DIV that proceeded until 3 DIV, were the lowest value was obtained (0.5-fold *vs*. NS, $p<0.01$). Despite the elevation next observed at 7 DIV, and although no significant differences, the values were still lower than those obtained in NS (0.7-fold *vs*. NS). Most interesting, although the GL261 cells have shown the highest values for Mrp1 expression they were not more elevated than the levels obtained in NS. The results, therefore, indicate that NS are the cells that most closely resemble the glioma cells in terms of the Mrp1 expression.

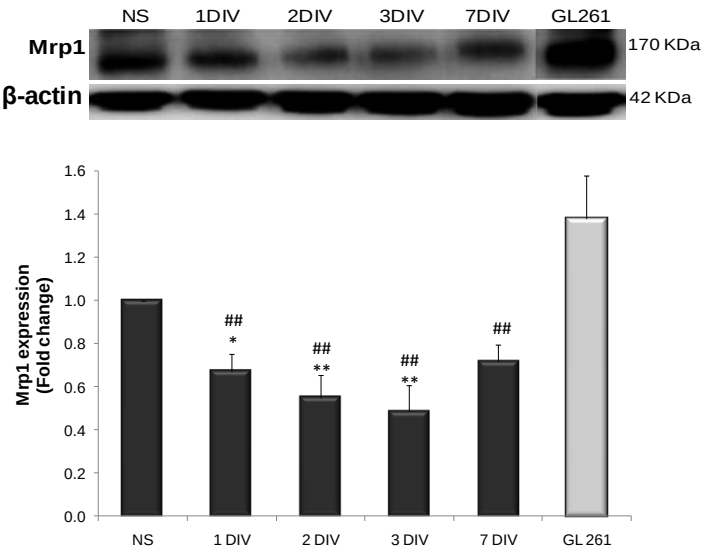


Fig. III.6 – Comparison of Mrp1 expression in neurospheres and differentiating astrocytes with that in glioma cells. Cells were cultured as indicated in methods. Total cell lysates were analyzed by Western blot with antibody specific for multidrug resistance protein 1 (Mrp1). Representative results from one experiment are shown. Similar results were obtained in at least three independent experiments. The intensity of the bands was quantified by scanning densitometry, standardized with respect to β-actin protein and expressed as mean ± SEM fold change compared to naurospheres (NS). *p<0.05 and **p<0.01 *vs.* NS; $^{\#\#}$p<0.01 *vs*. GL261 cells. DIV, days *in vitro* under differentiating conditions.

2.2 Cells differentiated during 24 h from NS are those exhibiting autophagic levels most similar to the ones observed in glioma cells

Autophagy is an intracellular lysosomal degradation process, which plays an important role in cell growth and development (Chen *et al*., 2012). Autophagic activity evidenced to be decreased in glioma stem/progenitor cells (Zhao *et al*., 2010) and when induced demonstrated to turn cells more sensitive to radiotherapy (Zhuang *et al*., 2012). LC3 conversion (LC3-I to LC3-II, expressed by LC3II/I ratio) was assayed by Western blot, given their different mobilities, as a biomarker of the autophagy. While LC3-I is cytoplasmic, LC3-II is autophagosome membrane-associated (Kabeya *et al*., 2000; Mizushima, 2007). Autophagic activity was assessed in GL261 and in differentiating cells from NS, using an anti-LC3 antibody, and the results normalized against β-actin levels (Fig. III.7). Conversion of LC3-I to LC3-II demonstrated to increase from NS to 7 DIV differentiated cells (2.8-fold *vs.* NS). Although only one experiment was accomplished, and based that GL261 cells showed a 2.1-fold increase in LC3II/I expression, as compared to NS, we may hypothesize that similarities between GL261 and differentiating cells are better at 1DIV cells that also evidenced a 2.2-fold *vs*. NS. Thus, in what concerns the claimed decreased levels of autophagy in glioma cells equivalent values were observed in cells after 24 h of differentiation from NS (1 DIV).

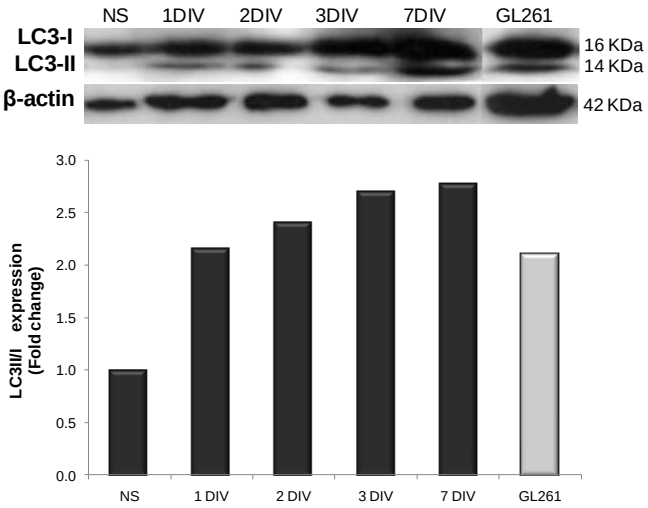
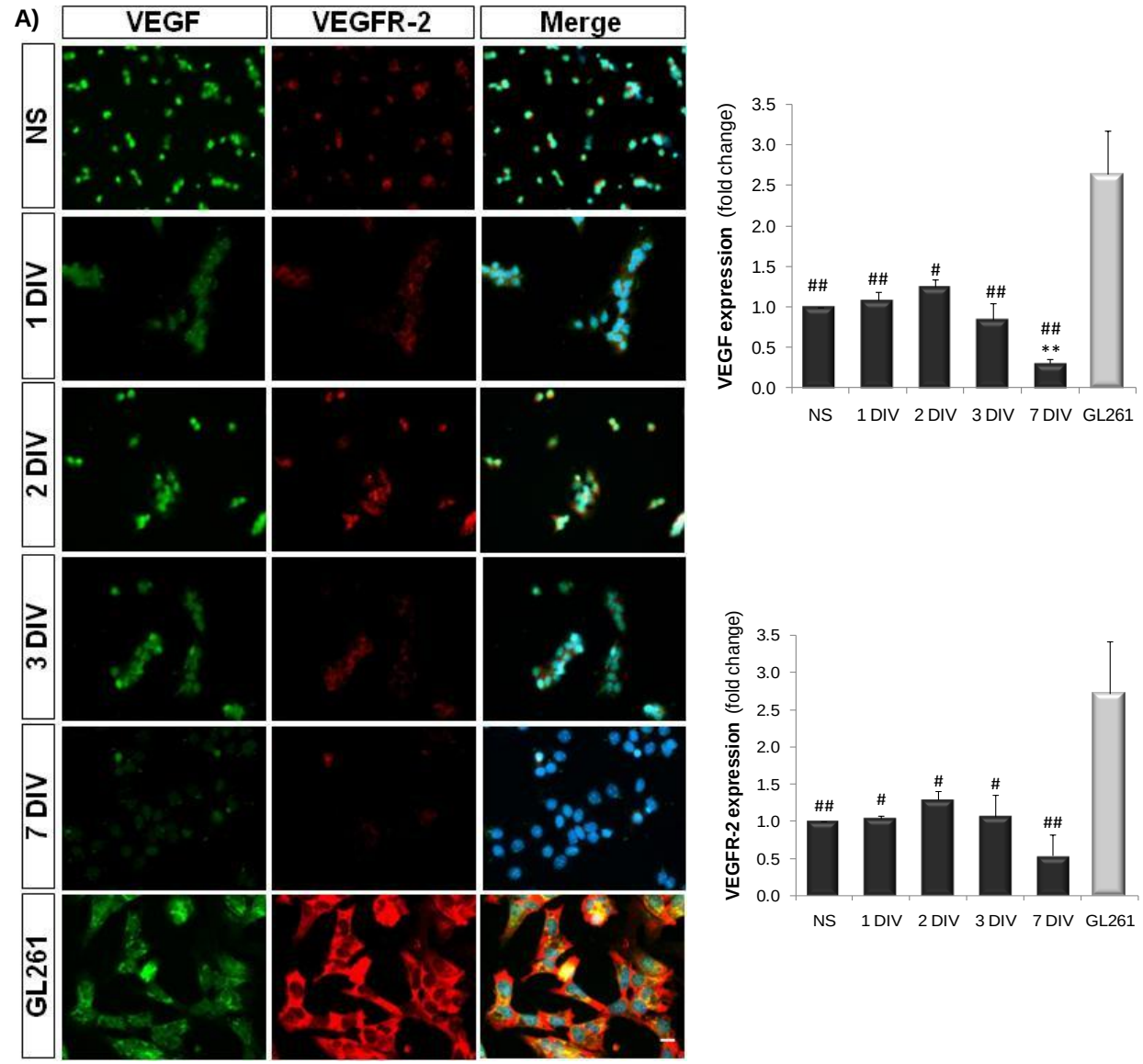


Fig. III.7 – Comparison of the LC3-I to LC3-II expression conversion between the different stages from neurospheres to differerentiated astrocytes and glioma cells. Cells were cultured as indicated in methods. Total cell lysates were analyzed by Western blot with an antibody specific for microtubule-associated protein light chain 3 (LC3). The intensity of the bands was quantified by scanning densitometry, standardized with respect to β-actin protein and expressed as fold change *vs*. neurospheres (NS). Data was obtained from only one experiment. DIV, days *in vitro* under differentiation.

2.3 Glioma cells evidence higher levels of VEGF and VEGFR-2 expression than NS or differentiating astrocytes

The expression level of VEGF in NS, differentiating cells and GL261 cells was determined by both Western blot and immunocytochemistry, using an antibody against VEGF. The expression level of VEGFR-2 (vascular endothelial growth factor receptor 2) was determined through immunocytochemistry, using an antibody against VEGFR-2 (Flk-1). As shown in Figure III.8A the staining for VEGF (green) is mostly nuclear and the intensity of fluorescence changes along differentiating days *in vitro,* with values more elevated in NS and in the first stages of cell differentiation, peaking at 2 DIV (1.3-fold *vs.* NS) although no significant differences. The lowest values were observed at 7 DIV, representing about 50% of the one obtained in NS ($p<0.01$). GL261 cells, in contrast, exhibited 2.6-fold higher values than NS ($p<0.01$), attesting its angiogenesis potential and glioma growth abiliy. A similar profile was obtained for VEGFR-2, the most important receptor in the direct regulation of angiogenesis and expressed at high levels in glioma (Sharma and Saad, 2011), as depicted in Figure III.8A. Staining of VEGFR-2 (red) is mainly cytoplasmic and like VEGF the highest value was observed at 2 DIV cells (1.3-fold *vs.* NS) with the 7 DIV cells presenting the lowest value (less than 50% of NS, $p<0.01$), but even so always less than a half of the expression in GL261 cells (2.7-fold *vs*. NS, $p<0.01$) and therefore, none of the several stages of differentiation or NS was even close of the VEGF and VEGFR-2 values obtained in GL261. However, although significantly different ($p<0.05$), the expression for both VEGF and VEGFR-2 at 2 DIV cells was the one closer to GL261 cells. Curiously, as the immunocytochemistry assay evidence, a higher expression of VEGFR-2 than VEGF was obtained in NS and progenitor phenotypes.

Despite the reduced number of experiments (n=2) and the elevated standard error of the mean (mainly for GL261 experiments) the VEGF expression by Western blot revealed a profile similar to the immunocytochemistry assay (Fig. III.8B). However, this assay revealed values of VEGF in GL261 almost 3 times more elevated than those in the previous immunocytochemistry determination (7.6-fold *vs.* NS).



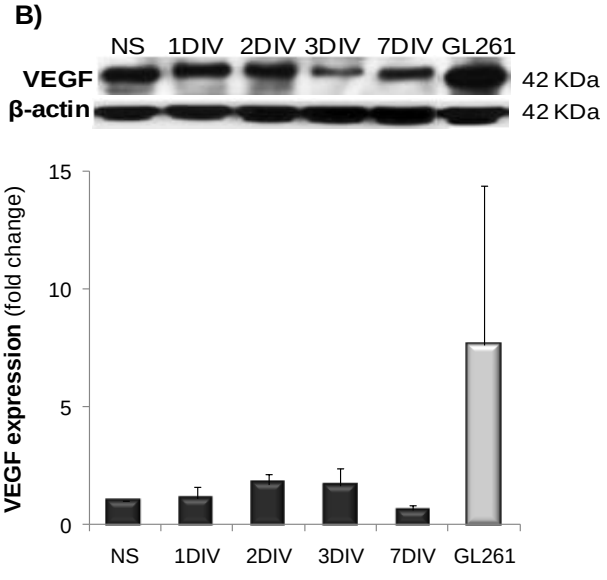


Fig. III. 8 – **Comparison of VEGF and VEGFR-2 expression between neurospheres plus the astrocytes differentiation stages and the glioma cells.** Cells were cultured as indicated in methods. A) Nuclei were stained with Hoechst dye (blue) and cells labeled for vascular endothelial growth factor (VEGF, green) and vascular endothelial growth factor receptor 2 (VEGFR-2, red). Graph bars represent the fold changes (mean ± SEM) for positive VEGF and VEGFR-2 cells, relatively to the total number of nuclei. Data obtained from at least three independent experiments. Scale bar represents 300 µm. *p<0.05 and **p<0.01 *vs.* neurospheres (NS); $^{\#}$p<0.05 and $^{\#\#}$p<0.01 *vs*. GL261 cells. B) Total cell lysates were analyzed by Western blot with specific antibody for VEGF. The intensity of the bands was quantified by scanning densitometry, standardized with respect to β-actin protein and expressed as mean fold change compared to NS, assumed as 1. Data was from only two experiments. DIV, days *in vitro* under differentiation.

2.4 MMP-9 and MMP-2 expression levels in glioma cells are more similar to the ones in 1 and 7 DIV differentiating astrocytes

The concentration of MMP-9 and MMP-2 in NS, differentiating astrocytes and GL261 cells was determined by gelatin zymography (Fig. III.9A). Results were normalized to the correspondent total protein concentration (Figs. III.9B and C). The activity of MMP-9 evidenced maximal levels in the differentiating cells at 2 DIV (1.9- fold *vs*. NS, $p<0.01$) decreasing thereafter until 7 DIV (1.6-fold *vs*. NS, $p<0.01$). The lowest values for MMP-9 activity were seen in NS that evidenced to significantly differ from the levels obtained in GL261 cells (1.8-fold *vs*. NS, $p<0.05$). All the other results obtained were higher than those in GL261 and, although only significantly increased at 2 and 3 DIV. Again, but now for the activity of MMP-2, the lowest value was also observed in NS that significantly differed from all the other differentiating temporal windows and even glioma cells that showed to be 1.8-fold enhanced as compared to NS ($p<0.05$). The highest level was obtained at 3 DIV (2.4-fold *vs*. NS, $p<0.01$), which was again superior to that of MMP-2 in GL261 cells.

In summary, the 1 and 7 DIV differentiated astrocytes were the ones closer to the GL261 cells in what concerns to both MMP-2 and MMP-9 levels.

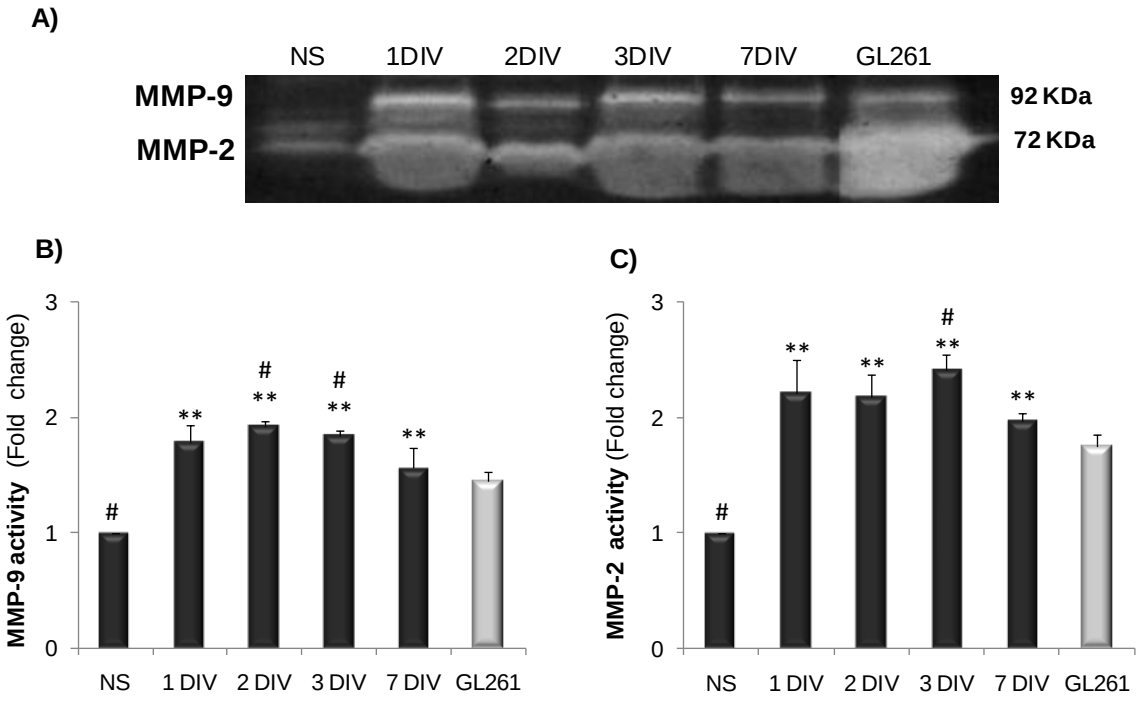


Fig. III.9 – Comparison of matrix metalloproteinase-9 (MMP-9) and MMP-2 activities between neurospheres plus the astrocytes differentiation stages and the glioma cells. Cells were cultured as indicated in methods. Conditioned media was collected from neurospheres (NS) and from all cells at the several differentiating windows, as well as from GL261 cells line for quantification of MMP activity. A) MMP-2 and MMP-9 were identified by their apparent molecular mass of 72 and 92 kDa, respectively. Representative results from one experiment are shown. B) and C) Graph bars represent the intensity of the bands quantified by scanning densitometry and standardized with respect to protein quantification for MMP-2 and MMP-9, respectively. Results are expressed as mean ± SEM from at least three independent experiments, and presented as fold change relatively to NS, assumed as 1. **$p<0.01$ *vs.* NS; $^{\#}p<0.05$ *vs*. GL261 cells. DIV, days *in vitro* under differentiation.

2.5 Evaluation of S100B protein expression by NS/differentiating astrocytes and its comparison with glioma cells

The extracellular concentration of S100B in NS, differentiating astrocytes and GL261 cells was determined by ELISA, and normalized to the respective total protein concentration. Results depicted in Figure III.10 evidence that the release of S100B by NS is almost null (~0.1nM), in opposite to all the other results found, which revealed to be markedly more elevated. However, with the exception of the values obtained for 2 DIV cells ($p<0.01$ *vs*. NS) all of them were significantly lower than the ones exhibited by the GL261 cells (~1µM). These cells evidenced the highest amount of S100B released to the extracellular media and the concentration obtained from the 2 DIV cells ($p<0.05$ *vs.* GL261) was the one closer to GL261 cells.

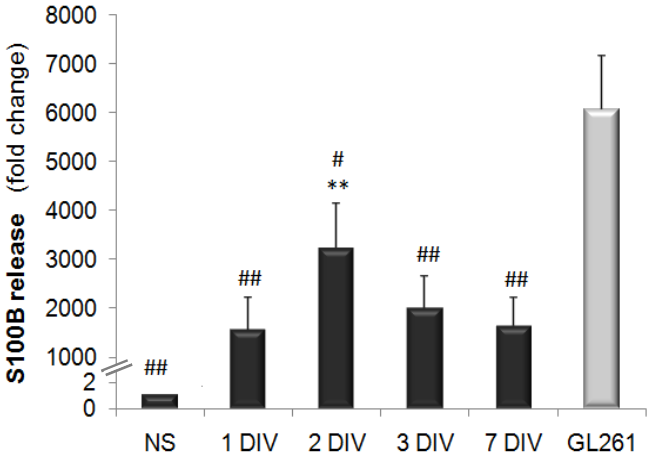


Fig. III.10 – Comparison between S100B release levels from neurospheres, differentiating astrocytes and glioma cells. Cells were cultured as indicated in methods. Conditioned media was collected from neurospheres (NS) and from all cells at the several differentiating windows, as well from GL261 cell line for quantification of S100B concentration by ELISA assay. Graph bars represent the extracellular concentration of S100B protein expressed as mean ± SEM from at least three independent experiments, and presented as fold change compared to NS, assumed as 1. **$p<0.01$ *vs.* NS; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ *vs*. GL261 cells. DIV, days *in vitro* under differentiation.

2.6 Migratory potential of NS and differentiating astrocytes are higher than that of glioma cells

The migratory capability was evaluated through the Boyden chamber assay described in section II.3.7. Here, two different parameters were evaluated, answering to two different questions. Firstly, it was determined the migratory properties of NS, as well as 1 DIV and 7 DIV differentiating astrocytes, and GL261 cells towards VEGF, a growth factor described as an important chemoattractant present in the tumour mass. Using this experimental design we evaluated the developmental stage that evidenced the migratory potential most similar to GL261 cells. Secondly, it was evaluated the ability of NS, as well as 1 DIV and 7 DIV differentiating astrocytes to migrate towards GL261-conditioned media. In this case, we will investigate which developmental stage present the higher tropism to glioma cells.

We start by evaluating the most suitable cell concentration to be used in the migration assay. Thus, two different cellular concentrations were tested: 1.5×10^4 cells/well and 3.0×10^4 cells/well. First concentration has revealed to be the most adequate because at higher cell number the NS easily form aggregates, which prevent them from crossing the membrane pores, thus forming visible blots on the upper face of the membrane (results not shown). It was also tested which VEGF concentration

was the most suitable to be used in the studies. It were tested 1, 2.5, 5, 10 and 20 ng/mL concentrations, and that of 20 ng/mL VEGF concentration was the one selected for the assays (results not shown). Regarding the evaluation of migratory potential towards VEGF (Fig. III.11A), we observed no differences between NS and the differentiating cells but unexpectedly GL261 cells evidenced the lowest ability when compared to the first, although no significant differences (Fig. III.10B). A slightly higher migratory capacity was shown by 1 DIV cells (ns). These cells were also the ones that exhibited the greater capacity to migrate towards GL261-conditioned as depicted in Figures III.11A and C.

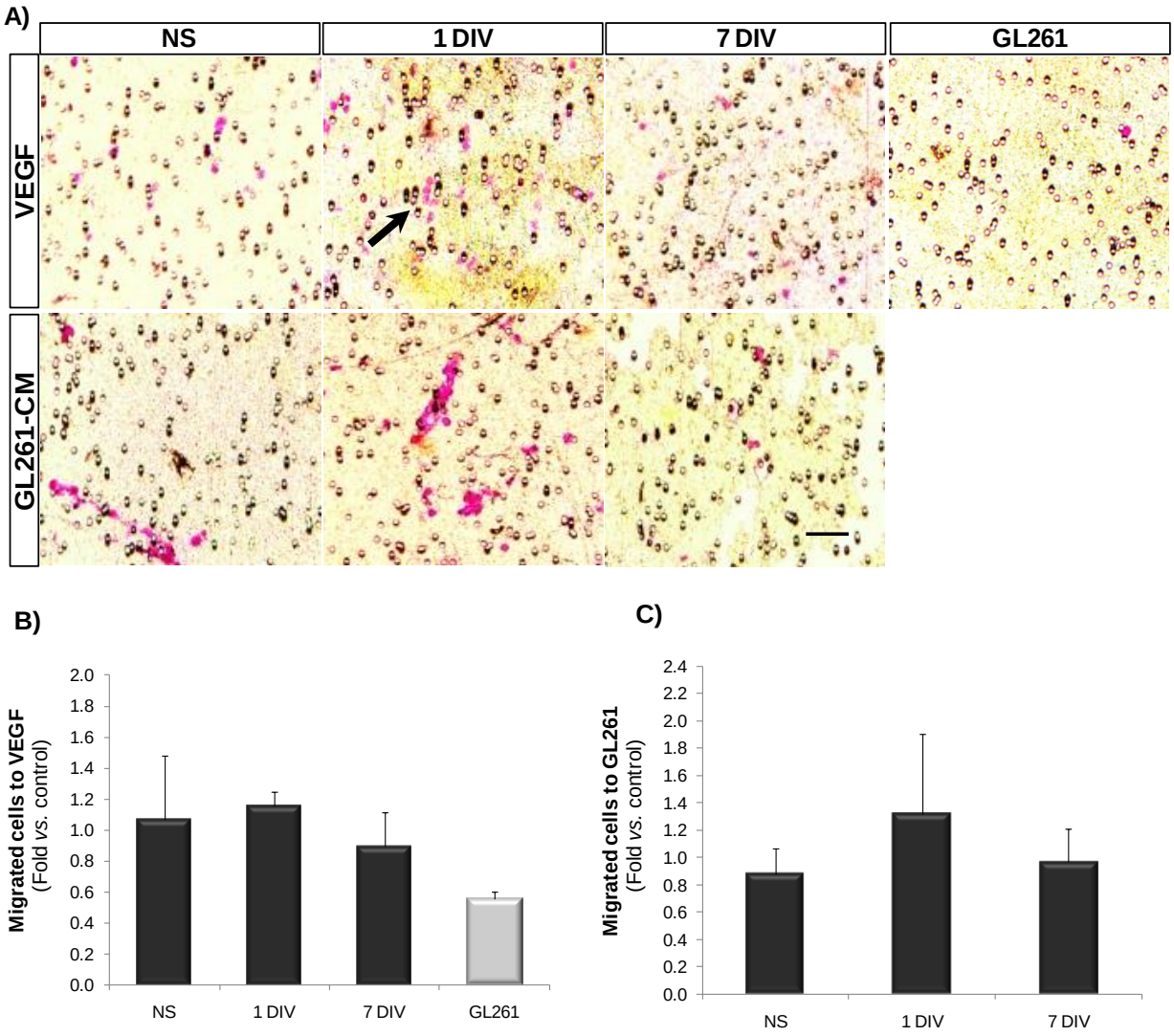


Fig. III.11 – Comparison of the migratory potential between neurospheres plus the astrocytes differentiation stages and the glioma cells towards VEGF and GL261-conditioned media. Cells were cultured as indicated in methods. Migration was performed using a Boyden Chamber and the cells migrated for 5 h, crossing a PVP-treated polycarbonate filter (8 µm pore size) as described in methods. Representative results of one experiment evidencing the migrated cells (pink, indicated by black arrow), are shown in (A). The total number of cells per well that migrated to 20 ng vascular endothelial growth factor (VEGF) (B) and to GL261-conditioned media (GL261-CM) (C) were counted and the results expressed as fold change *vs*. control. Results are mean (± SEM) from at least three independent experiments performed in triplicate. Scale bar represents 100 µm. DIV, days *in vitro* under differentiation.

IV. DISCUSSION

NSC can be isolated from the fetal or adult brain and expanded in culture through NS, which have the ability to generate neurons, astrocytes and oligodendrocytes (Sergent-Tanguy *et al*., 2006; Sun *et al*., 2011; Suzuki *et al*., 2004). Our results show that the cellular mass obtained from primary culture of cortex fetuses at E15, when maintained in proliferation conditions with growth factors, give rise to the formation of NS. As previously described by some groups, the data obtained from the immunocytochemistry assays performed in the present study evidence that NS are really heterogenic as they are composed by a mixture of both undifferentiated and differentiated cell types (Bonnamain *et al*., 2012; Breier *et al*., 2010; Garbossa *et al*., 2012).

It is described that Sox2 and Nestin are the proteins most expressed by NS as cell markers of undifferentiated cells (Abe *et al*., 2006; Bani-Yaghoub *et al*., 2006; Brunet *et al*., 2004), thus confirming these cells as NSC. Likewise, Sox2- and Nestin-positive cells are also BrdU-positive (Bani-Yaghoub *et al*., 2006; Sun *et al*., 2011). Results here presented are consistent with those observations, since our NS cultures express high levels of Sox2 and Nestin, being the two most representative cell markers at this stage of development. Associated to their proliferative potential, BrdU also had a high level of staining in NS, confirming that these undifferentiated cells have the capability to maintain cellular self-renewal and expansion. The BrdU-positive cells had a percentage most similar to Nestin-positive cells, and lower than the Sox2-positive cells. These results are in agreement with data from Sun *et al*. (2011) showing that BrdU-positive cells are also nestin-positive cells, and from Bani-Yaghoub *et al*. (2006) evidencing that not all Sox2-positive cells are BrdU-positive ones. Besides revealing the undifferentiated cell markers, NS also show a small amount of markers characteristic of differentiated cells. Thus, we found that NS express astroglial-markers, such as vimentin which is specific for glial progenitor cells, GLAST that is expressed by early progenitor astrocytes (and other glial cells) and lately by mature astrocytes, as well as GFAP that is expressed in differentiated astrocytes or in differentiation. However, vimentin has a higher expression level in NS than GFAP or GLAST, leading us to conclude that there is an elevated percentage of early progenitor astrocytes in NS. These data is supported by previous results describing high levels of vimentin in NPC (Chan-Ling *et al*., 2009), and Sox2 and Nestin co-expression (Li *et al*., 2011). Preceding studies have demonstrated GFAP expression on these floating aggregates from several mouse brain tissues (Brazel *et al*., 2005; Breier *et al*., 2010; Rieske *et al*., 2007), which are in accordance with our own results. Regarding GLAST-positive cells, our data is similar to that of Brunet *et al*. (2004) that also show GLAST expression in NS. Since earlier studies showed a decrease of vimentin expression when GFAP expression increase (Chan-Ling *et al*., 2009; Liu *et al*., 2002), and given that GLAST stain early progenitor astrocytes, their sequential order of expression is: first the vimentin marker, followed by the GLAST marker and finally the GFAP one as we found, since we obtained in NS a higher percentage of vimentin expression, followed by GLAST and finally the GFAP expression, the less pronounced astrocytic cell marker.

The immunostaining of oligodendrocytic cells in our NS culture indicated the presence of oligodendroglial progenitors (NG2-positive cells) but not of oligodendroglial mature cells (MBP-positive cells). The presence of NG2-positive cells in NS is also described in the literature (Brazel *et al*., 2005; Machon *et al*., 2005) and indicated as corresponding to near 10%, a value very similar to that obtained by us. The absence of mature oligodendrocytes in NS is not surprising as it is known that maturation

of oligodendrocytes takes place only in early postnatal life (Baumann and Pham-Dinh, 2001), a stage where cells are already differentiating instead of being in a proliferation stage.

Finally, the evaluation of neuronal cells in our NS culture indicated that the presence of neuronal progenitor cells (HuC/D-positive cells) is higher than neuronal mature cells (MAP2-positive cells). Surprisingly, there is a lack of information in the literature regarding the presence of HuC/D- and MAP2-positive cells in NS, although some authors point to the presence of βIII-Tubulin-positive cells (or Tuj1-positive cells) in NS (Breier *et al*., 2010; Sun *et al*., 2011; Suzuki *et al*., 2004), and, particularly, in human NSC (Rieske *et al*., 2007). Since βIII-Tubulin is a marker of neuronal cells, these previous studies corroborate ours. Thus, βIII-Tubulin expression should be confirmed in our cultured NS in the future.

Breier *et al*. (2010) demonstrated that undifferentiated and differentiated markers are differently distributed in the NS. They showed that the nestin-positive cells are located in the periphery of the NS, while βIII-Tubulin-positive cells and GFAP-positive cells reside in the center, due to their location in the core of neurosphere, where cells do not contact (or have a poor contact) with undifferentiation conditions, leading them to differentiate. Thus, it should be interesting to additionally analyse our NS in a confocal microscope in order to study the cellular distribution and location of differentiated and undifferentiated cells on these floating aggregates.

The change of culture media from a proliferative inducer into a differentiate one led to an alteration of the protein expression profile. Our results show that in differentiation conditions *in vitro*, cells adopt a characteristic astrocyte-like morphology, with large polyhedral cytoplasm and small processes. These data are consistent with the work described by Brunet *et al*. (2004) in which this differentiation method was developed. Comprehensively, the expression levels of differentiated cell markers increase along differentiation into astrocytes, particularly the astrocytic markers, while those of undifferentiated ones decrease. The highest expression levels along differentiation belong to GFAP and GLAST markers since these are specific astrocytes markers. Also, their increase is accompanied by the reduction of vimentin expression levels. Our results are supported by previous studies in which it was found that the low levels of GFAP and GLAST expression in NSC, increase with differentiation (Brunet *et al*., 2004; Sergent-Tanguy *et al*., 2006). However, like in our work, some cells co-express GFAP and vimentin (Li *et al*., 2011), corresponding to glial cells (Quinlan and Franke, 1983). Curiously, the co-expression of vimentin and GFAP was found in all grades of malignancy, such as in gliomas (Herpers *et al*., 1986; Quinlan and Franke, 1983). Sox2 appears to be the marker with the third highest expression level in differentiating cells and it is maintained visible in the immunostaining assays during all days of differentiation, even when the number of mature astrocytes increases. Similarly, although less expressed, also nestin was detected along all the differentiation process. This may have happened because some astrocytes express Sox2 (Jinno, 2011) and GFAP-positive cells can still co-express a low level of nestin during development and differentiation (Brunet *et al*., 2004; Sergent-Tanguy *et al*., 2006) because, according to Sergent-Tanguy *et al*. (2006), differentiated cells might retain NSC properties and then express progenitor markers. Interestingly, these same Authors argue that due to the late expression of nestin during maturation procedure, nestin-postive cells in population tend to aggregate (Sergent-Tanguy *et al*., 2006). Apparently our results are in accordance

with this assumption since we observed that the individualization of the cells occurs in parallel with the loss of clusters along the last days of differentiation, also corresponding to a decrease in nestin-expression.

Overall, our culture conditions thus favored astrocyte differentiation, once the expression of neurons and oligodendrocytes markers are decreasingly observed and inversely correlated with the ones of astrocytes along differentiation. Accordingly with Brunet *et al*. (2004) and Breier *et al*. (2010), the removal of growth factors, particularly the EGF one, prevents the maintenance of the cells as undifferentiated, while, the addition of FBS instead leads to differentiation of stem cells into astrocytes (Abe *et al*., 2006; Sakai *et al*., 1990), therefore increasing the levels of GFAP (Brunet *et al*., 2004; Jinno, 2011). The differentiation into oligodendrocytes is inhibited by the presence of FBS in media, due to the presence of the bone morphogenetic protein (BMP) in the serum which belongs to the major class of the transforming growth factor β superfamily, known to promote the selective differentiation of astrocytes (Mabie *et al*., 1997; Wang *et al*., 2012). Moreover, it is described that while Sox2 overexpression allows the differentiation of progenitors into astrocytes, it inhibits neurogenesis (Bani-Yaghoub *et al*., 2006). This concept further supports our data, because the high Sox2 expression along differentiation could have indeed prevented the neuronal differentiation leading to the observed low levels of neuronal markers.

Regarding all of these results, it will be tempting to perform, in the future, the evaluation of the markers here used in the characterization of NS and differentiating cells by flow cytometry (GUAVA), to confirm the data obtained given its highest specificity.

As previously mentioned, gliomas are formed by a heterogenous mixture of several glial phenotypes, composed simultaneously by immature cell types, poorly differentiated astrocytes and mature cells (Park and Rich, 2009; Siebzehnrubl *et al*., 2011). Similarly, NS and first days differentiated cultured NS into astrocytes are also composed by a heterogenic cellular mixture, and as so we may talk about some degree of resemblance between glioma cells and differentiating-astrocytes.

Several studies have been developed to understand the similarities between glioma cells and NSPC, suggesting that the transformation of NSC or NPC first can be in the origin of gliomas. According to some recent works, modifications in the normal course in the differentiation of glial NPC may lead to the generation of abnormal cells, instead of mature astrocytes (or oligodendrocytes) (Sanai *et al*., 2005). Thus, in the present work, the expression of some tumour-related factors was evaluated in primary NS, differentiating-astrocytes and GL261 cells, in order to investigate which developmental stage, from NS to differentiating astrocytes, had the highest tumourigenic potential.

The multidrug resistance phenomenon can be mediated by several ABC transporters (Kondo, 2006; Sutter *et al*., 2007), and regarding tumours, it is one of the main causes of treatment failure and disease progress. Mrp1, the best studied of the nine members of Mrp family (family of ABC transporters), has been detected in gliomas cell lines (Bart *et al*., 2000; Calatozzolo *et al*., 2005), as well as Mrp3 (with structure similarities to Mrp1 protein), a protein that Kuan *et al*. (2010) showed to be highly expressed in human GBM but not in normal brain cells. Moreover, Mrp1 expression, that revealed to be significantly up-regulated in cancer stem-like cells (Jin *et al*., 2008) and in $CD133^{+}$

human brain glioma stem cells (Bi *et al*., 2007), can be one of the reasons why tumour cells resist to chemotherapeutic drugs, being suggested that chemosensitization of cells with Mrp1 inhibitors might increase the efficacy of the usual glioma treatment (Peignan *et al*., 2011). Intriguingly, undifferentiated NPC also show high levels of ABC transporters expression (Islam *et al*., 2005a; Islam *et al*., 2005b). These observations are consistent with our findings showing higher Mrp1 levels in NS and GL261 cells. Recently, Jin *et al*. (2010) by comparing NSC and CSC found that both had high Mrp1 expression level. While the high levels in NS or in NSPC are necessary to their proliferation (Lin *et al*., 2006), in gliomas (particularly in glioma CSC) Mrp1 seem to be related with the resistance to multidrugs in the course of therapeutic methodologies (Dean *et al*., 2005; Salmaggi *et al*., 2006). In the differentiating-astrocytes this expression level begins to be lost with the increase of differentiation and consequent reduction of the number of undifferentiated cells, except in the last day of the differentiation process, where it occurs a high Mrp1 expression level. However these data were noticed by Calatozzolo *et al.* (2005) even in human normal mature astrocytes.

Autophagy is a subcellular process that degrades damaged organelles and proteins and involves a membranous organelle, the autophagosome (Liu *et al*., 2011; Rubinsztein *et al*., 2007). Autophagy has been implicated both in development and immunity (Chen *et al*., 2012; Levine and Deretic, 2007), and an abnormality in this autophagic process is related to malignant diseases. Therefore, autophagy can be both oncogenic and tumour suppressive, suggesting that the process can have different roles at the several stages of tumour development (Macintosh *et al*., 2012). Moreover, autophagy develops an important role in the regulation of self-renewal, differentiation, tumourigenic potential and radiosensitization of glioma-initiating cells, indicating that autophagy induction can promote the differentiation of these cells and their susceptibility to radiotherapy (Palumbo *et al*., 2012; Teres *et al*., 2012; Zhuang *et al*., 2011). Hence, to combat this phenomenon, it has been tested drugs that induce autophagy instead of apoptosis in malignant glioma cells (Kanzawa *et al*., 2004). Aoki *et al*. (2008) showed that LC3B (an autophagic marker) was expressed *in vivo* and *in vitro* glioma cells. Concerning embryonic development, some evidences support the existence of a complex interplay between autophagy and cell proliferation during mammalian neural development (Fimia *et al*., 2007). Moreover, Vazquez *et al*. (2012) referred that during differentiation, neuroephetilial cells undergo efficient remodel of their cytoskeleton and shape in an energy-consuming process, and since autophagy is required to recycle cellular components and provide energy, this mechanism could fulfill these requirements, supporting differentiation. Although it has been used a different autophagic marker, our results are coherent with these observations since the autophagic marker LC3B that we used evidenced to increase along differentiation, when cells begin to acquire a more specific fate. Surprisingly, the LC3B expression level in the second day of differentiating-astrocytes revealed to be increased as compared to glioma cells, probably as a consequence of the higher heterogenic population of differentiating cells that compose those phenotypes. In fact, glioma cell population evidenced a higher similarity to NS or one day differentiating-astrocytes. Nevertheless, these results need to be reassured by further experiments.

The activity of many MMP has been detected in the nervous system (Fujioka *et al*., 2012). MMP mediate the degradation of protein components of ECM in a variety of physiological and

pathological tissue remodeling processes, including embryo implantation, tumour invasion, metastasis, angiogenesis (Forsyth *et al*., 1999; Hagemann *et al*., 2012) and neovascularization (Sun *et al*., 2012). Levels of MMP-2 and MMP-9 are significantly elevated in injured brains (Fujioka *et al*., 2012). Sun *et al*. (2012) have verified that these two gelatinases are present in glioma specimens from patients and, most importantly, they were correlated, with the progression and the prognosis of these malignant tumours. Thus, the invasion ability of glioma is associated with the amount of MMP. Particularly, Thorns *et al*. (2003) demonstrated that besides MMP-2 and MMP-9 astrocytomas also exhibited immunostaining for, MMP-7, MMP-10 and MMP-11. Our results are somewhat consistent with the above described works. In fact, GL261 cells expressed MMP although we were expecting a higher level of expression, such as the one found by Hagemann *et al*. (2012) in GBM-cell lines. More commonly, MMP are abundantly expressed in NSC isolated from the human CNS (Frolichsthal-Schoeller *et al*., 1999) and play an important role in embryonic development (Ethell and Ethell, 2007). Fujioka *et al*. (2012) have shown that MMP might promote proliferation, neurite extension, and migration of newborn neurons in the developing brain. Wang *et al.* (2006) also relate MMP-2 and MMP-9 expression with neural progenitor cell migration and hypothesized that these gelatinases might play a role in providing an optimal niche for neural stem/progenitor cells. In the CNS, MMP-2 can be detected in various brain structures including astroglia and some neurons in the cortex, whereas the MMP-9 is expressed in the hippocampus, cerebellum, and cortex, predominantly in neurons (Fujioka *et al*., 2012). Such observations are consistent with our work since we found MMP-9 and MMP-2 expression in NS and in differentiating cells. We suggest that the increased activity of MMP-2, as compared to MMP-9, can derive from the increased expression of MMP-2 mainly in astroglia, the predominant phenotype of differentiating cells as already mentioned. Regarding MMP-9 expression, we have observed that an increased expression is present in NS till the second/third day of differentiating-astrocytes, decreasing thereafter. These results are supported by data from Fujioka *et al*. (2012), showing that the increase in the MMP-9 expression coincided with the phenotypes composed by NPC and with the increased representation of neuronal cells since MMP-9 expression mainly occurs in neurons. The decrease of MMP-9 in the more differentiated astrocytes stages can derive from the low percentage of neuronal cells representation. Overall, we believe that MMP should not be considered as the most reliable factor to ascertain about the developmental stage most similar to glioma, due to the activity variability along differentiation.

Regarding S100B, this protein is known to be involved in proliferation, differentiation and migration/invasion among other aspects. Previous studies have described that S100B is concentrated in astrocytes and in other glial cell types, which constitutively may release it (Yang *et al*., 1995; Zhang *et al*., 2011). S100B expression defines a late developmental stage after which GFAP-expressing cells loose NSC potential and ability to form NS and to acquire a mature developmental state (Raponi *et al*., 2007). Moreover, it is described that at nanomolar concentrations (as the ones observed in neurospheres), S100B exerts neurotrophic properties for normal brain development (Rothermundt *et al*., 2003). However, despite its paracrine/autocrine trophic effects at physiological concentrations, higher levels of S100B have shown toxic effects and have been detected in brain tumours (Michetti *et al*., 2012), particularly in astrocytomas (Zhang *et al*., 2011). Interestingly, such as MMP, S100B protein

is considered to promote cell invasion and migration, and to be related with the development of brain metastasis (Pang *et al*., 2012). S100B also contributes to tumourigenesis by inhibiting the function of the tumour suppressor protein p53 (Lin *et al*., 2004) and by regulating cell proliferation and differentiation through stimulation of mitogen protein kinases (Arcuri *et al*., 2005). According to these observations, Brozzi *et al*. (2009) found that S100B stimulates C6 glioma proliferation, also participating in normal astrocytes proliferation and activation through interactions with Src kinase. In our work we found a high release of S100B by GL261 cells (micromolar concentrations), corroborating data of the works previously referred. Such levels of S100B, described to increase cellular proliferation (Brozzi *et al*., 2009; Leclerc *et al*., 2007), suggest that S100B may contribute to reduce the differentiation potential of cells of the astrocytic lineage, then contributing to maintain an invasive phenotype. In contrast to our expectations of an increase in the S100B release along differentiation, as the cells loose NSC-potential and acquire a mature state, we only verified high levels in the second day of differentiating-cells. Therefore it highly recommendable to validate these results by using an alternative method of S100B evaluation, such as the immunoluminometric sandwich assay (ILMA) (Ghanem *et al*., 2001; Steiner *et al*., 2006).

The VEGF family is a potent regulator of angiogenesis and vascular permeability that uses tyrosine kinase receptors (in particular, VEGFR) to mediate its activities (Takahashi and Shibuya, 2005). VEGF not only promotes angiogenesis but is also a key factor in proliferation of malignant brain tumours (Sjostrom *et al*., 2011). This growth factor promotes tumourigenesis, via angiogenesis, of human glioblastoma stem cells, resulting in its rapid growth. Oka *et al*. (2007) showed that VEGF injection into the mouse brain leads to the massive expansion of vascular-rich GBM. Additionally, VEGFR-2 plays a crucial role in glioblastoma development (Sharma and Saad, 2011), prognosis, and response to therapy. Such as lately demonstrated by Sjostrom *et al*. (2011) the inhibition of VEGFR-2 by ionizing radiation increase tumour cell death. In astrocytomas, Knizetova *et al*. (2008) found that VEGFR-2 is coexpressed with VEGF, leading to tumourigenesis.

Our results seem to corroborate these observations since we have found high levels of VEGF and VEGFR-2 expression in GL261 cells. VEGF is also highly expressed by embryonic NSPC in culture and believed to contribute to developmental processes, including proliferation, differentiation, maturation, vascularization and neurogenesis (Kim *et al*., 2007; Roitbak *et al*., 2008; Shetty *et al*., 2005). In accordance, inhibition of the VEGF signaling reduces migration and induces differentiation (Joo *et al*., 2012; Kaus *et al*., 2010). These data are consistent with our findings evidencing an increase in VEGF and VEGFR-2 expression from NS to the second day of differentiating-cells, i.e. until a high number of undifferentiated cells is achieved, and a decrease when cells acquire a more differentiated state. This explains the study of Palmer *et al*. (2000) who showed that although expression of VEGF, as well as of its receptors, continues within the adult neurogenic zone, VEGF is not expressed in adult brain, out of these areas. Therefore, the reduction of VEGF and VEGFR-2 in the last day of differentiation, as in the cited work, may derive from cell-maturation inducing their decrease. Interestingly, we have verified a higher expression of VEGFR-2 than of VEGF in NS and progenitor phenotypes.

These enhanced levels of VEGFR-2 expression can be related to results obtained in our work regarding migration. Recent studies indicate that tumour-upregulated VEGF acts as a soluble chemotactic factor, by inducing tropism of NSC (mainly to glioma) (Colleoni and Torrente, 2008; Joo *et al*., 2012). Considering our results, the first day of differentiating-cells was the stage evidencing the highest VEGFR-2 expression together with the most elevated migratory ability. This suggests that cells with higher VEGFR-2 expression are attracted more easily to VEGF than those with lower levels of expression. Nevertheless, the migration tendency of NSC towards gliomas, demonstrated by several *in vivo* studies, is mediated not only by VEGF but also by growth factors, cytokines and chemokines secreted from malignant glioma (Heese *et al*., 2005; Koizumi *et al*., 2011). Thus, although we have not obtained a high level of migration when cells were submitted to attraction by GL261-conditioned media, we believe that they were attracted not only by VEGF but also by other additional factors. In both cases, highest migration level was observed in the first day of differentiating-cells. These findings can be explained by the work of Ehtesham *et al*. (2004) suggesting that tumour-tropic NSC exhibit an astrocytic precursor phenotype. The lower migratory level exhibited by cells at the last day of differentiation somehow reinforces the concept that progenitor cells are the ones retaining the ability to travel through mature parenchyma. Thus when the nestin-expression is lost, cells loss the migratory ability and that when the nestin-expression disappears, cells lose its migratory ability. In fact, Sanai *et al*. (2005) have shown that in the injured adult brain, nestin-positive cells really migrate to the site of injury from the SVZ, indicating that progenitor cells retain the ability to travel through mature parenchyma. Thus, cells at the first days of differentiation are those behavioring more similarly as they have increased number of astrocytic precursors and high nestin-expression levels. Surprisingly, the migratory ability of GL261 cells was lower than what we expected. Indeed, in some previous studies it was indicated that malignant gliomas have high migratory capacity (Demuth and Berens, 2004; Lefranc *et al*., 2005; Sanai *et al*., 2005). However, the absence of migratory characteristics was ascertained in the GL261 cell line (Benetti *et al*., 2000), which might explain our results obtained in this cell line. Nevertheless, it is important that these migration studies will be reinforced by the application of other methodologies, such as the migration fluorimetric assays (Eccles *et al*., 2005; Hong *et al*., 2008; Hsieh *et al*., 2011). In addition, we believe that it should be important to quantify the content of the conditioned media from glioma cells in terms of VEGF concentration, by using ELISA methodology, in order to perform migration assays both in NS and in the first day of differentiating-cells. Moreover, once it is well known the ability of the glioma cells to release monocyte chemotactic protein 1 (MCP-1) (Bottazzi *et al*., 1983; Kuratsu *et al*., 1989; Meltzer *et al*., 1977) and since it has been shown that this chemokine, expressed both *in vivo* and *in vitro* conditions in brain tumour cells, has the ability to chemoatract NPC (Magge *et al*., 2009), it will be interesting to investigate the migration of both NS and differentiating-astrocytes towards this protein.

In summary, using our *in vitro* model of neural stem/precursor cells proliferation and differentiation, the main concluding remarks of this study are that: a) the methodology used to promote differentiation of NS into astrocytes was effective since after seven days *in vitro,* with the described growth media, cells acquired properties of mature astrocytes; b) first and second days of differentiating astrocytes evidence the closest similarities to GL261 cells, regarding some tumour-related factors.

Together we conclude that these phenotypes show a great potential to be in the origin of gliomas. The main results and conclusions obtained in this study are summarized in Fig.IV.1.

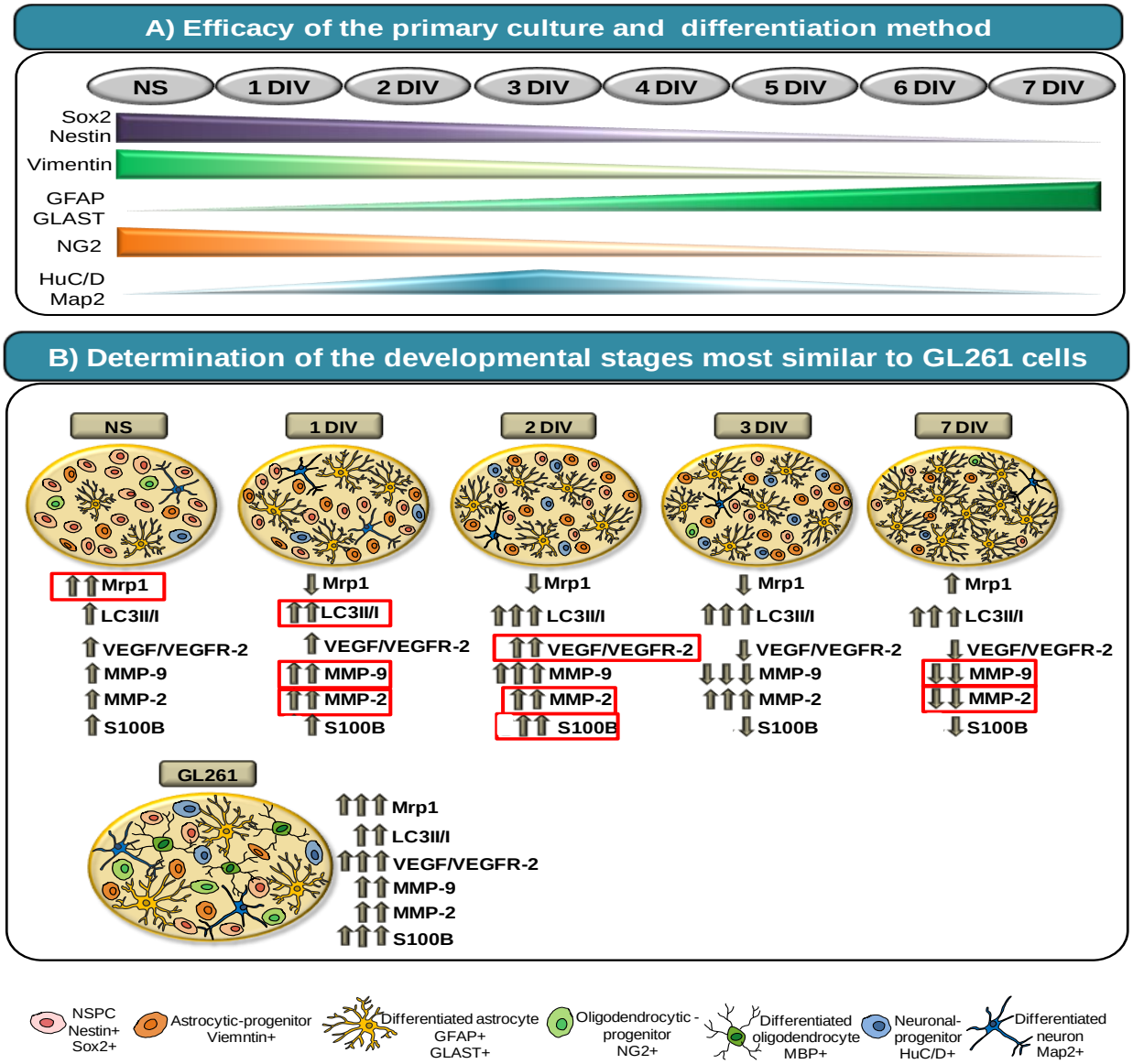


Fig. IV.1 – Summary of the main results and conclusions from the study. A) Representation of cellular markers along differentiation. The gradual increase or decrease of colors (ranging from light to dark, or vice versa) for each marker, corresponds, respectively, to an increase or decrease of its expression in neurospheres (NS) and in cell days *in vitro* under differentiation conditions (DIV). Throughout differentiation there is a decrease of Sox2- (that stains stem/progenitor cells), nestin- (stem/progenitor cells), vimentin- (progenitor glial cells), and NG2- (oligodendroglial progenitors) positive cells, in parallel with an increase in GFAP- (glial fibrillary acidic protein, mature/differentiated astrocytes) and GLAST- (glutamate aspartate transporter, differentiating astrocytes) positive cells. In addition, there was an increase of HuC/D- (neuronal progenitors) and MAP2- (microtubule-associated protein 2, mature/differentiated neurons) positive cells until the 3 DIV, followed by a decrease till the 7 DIV. B) Representation of the tumour-related factors expression in NS, differentiating astrocytes and GL261 (glioma cell line) cells. The gray arrows represent the level of factor expression; sequentially, three arrows indicate the highest expression level), two arrows (the intermediate expression level), and one arrow (the lowest expression level). Arrows pointing upwards represent the increase of expression relatively to the previous DIV, while arrows pointing down represent the decrease of expression relatively to the previous DIV. The red squares symbolize, for each evaluated factor, the value (and the phenotype) most similar to the level of expression in GL261 cells. Overall, the phenotypes with major similar factors when compared to GL261 cells, are the 1 and 2 DIV differentiating astrocytes. MMP, matrix metalloproteinasexs; Mrp1, multidrug resistance-associated protein 1; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor 2.

Future perspectives

According to the literature, cells can express more tumour-related factors when involved in the tumoural mass; thus, we intend to plate our cells (NS and differentiating astrocytes) in GL261-conditioned media. This methodology will allow us to observe whether NS and differentiating astrocytes in the presence of glioma cells, express higher levels of tumour-related factors, namely the parameters evaluated by us in the present work, than in their growth media.

It would be also interesting, as initially intended, to silence some genes which are thought to be more involved in tumourigenesis, such as PTEN and p53 (Zheng *et al*., 2008), in both NS and differentiating cells, thus inducing a malignant transformation of these cells. Then, we aim to evaluate which transformed phenotype will be closer to the phenotypr and will acquire the same tumourigenic properties of glioma cells. Additionally we also intend to evaluate the expression of CD133-positive cells, in order to identify the percentage of tumour-initiating cells on these phenotypes and on glioma cells. The CD133 is a neural stem marker, and therefore it will identify the brain tumour stem cells. In fact, it has been described that GBM have abundant clusters of CD133-positive cells, which are called the glioblastoma stem cells (Christensen *et al*., 2011; Pfenninger *et al*., 2007).

Finally, since in this work we have studied the migratory capability of NS and differentiating-astrocytes towards glioma cells, we intend to further explore the potential of these cells in glioma treatment. Thus, we additionaly aim to develop drug-loaded lipid nanoparticles that will be incorporated in these cells, in an attempt to use these systems as a promising tool to efficiently deliver drugs into the brain tumours.

In summary, the important issues that need to be explored in the future are the interaction between NS, differentiating astrocytes, and tumour cells, to better understand two particular points: (i) do these undifferentiating cells enhance or contribute to tumour growth and/or invasion ability? Are these cells suitable to deliver therapeutical compounds into gliomas? These are in fact important questions that surely deserve to be further exploited.

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